

This Page Is Inserted by IFW Operations  
and is not a part of the Official Record

## **BEST AVAILABLE IMAGES**

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images may include (but are not limited to):

- BLACK BORDERS
- TEXT CUT OFF AT TOP, BOTTOM OR SIDES
- FADED TEXT
- ILLEGIBLE TEXT
- SKEWED/SLANTED IMAGES
- COLORED PHOTOS
- BLACK OR VERY BLACK AND WHITE DARK PHOTOS
- GRAY SCALE DOCUMENTS

**IMAGES ARE BEST AVAILABLE COPY.**

**As rescanning documents *will not* correct images,  
please do not report the images to the  
Image Problem Mailbox.**

(19) World Intellectual Property Organization  
International Bureau



(43) International Publication Date  
27 March 2003 (27.03.2003)

PCT

(10) International Publication Number  
**WO 03/024425 A1**

(51) International Patent Classification<sup>7</sup>: **A61K 9/14, 38/28**

(21) International Application Number: **PCT/US02/29679**

(22) International Filing Date:  
19 September 2002 (19.09.2002)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:  
60/323,459 19 September 2001 (19.09.2001) US

(71) Applicant and

(72) Inventor: **McGURK, Simon, L.** [GB/US]; 1091 Penn Circle, Apt. G605, King of Prussia, PA 19406 (US).

(72) Inventors; and

(75) Inventors/Applicants (*for US only*): **MERISKO-LIVERSIDGE, Elaine** [US/US]; 258 Colwyn Terrace, West Chester, PA 19380 (US). **O'MAHONEY, Daniel** [IE/IE]; 75 Avoca Avenue, Blackrock County, Dublin (IE). **WEIDERHOLD, Amy** [US/US]; 5 Shannon Road, North Wales, PA 19454-4015 (US). **RAOOF, Araz** [IE/IE]; 58 Hornwood, Brennanstown Road, Cabinteely, Dublin (IE).

(74) Agents: **SIMKIN, Michele, M.** et al.; Foley & Lardner, Washington Harbour, Suite 500, 3000 K Street, N.W., Washington, DC 20007-5143 (US).

(81) Designated States (*national*): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, OM, PH, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM, ZW.

(84) Designated States (*regional*): ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, SK, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:

— with international search report

*For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.*

(54) Title: **NANOPARTICULATE INSULIN FORMULATIONS**

(57) Abstract: Nanoparticulate insulin compositions having effective an average particle size of less than about 5 microns are described. The compositions exhibit retention of insulin bioactivity, increased bioavailability, and increased consistency of bioavailability as compared to prior macro-sized insulin compositions. Methods of making nanoparticulate insulin compositions, dosage forms containing them, and the use of such formulations in insulin therapy are also described.



**WO 03/024425 A1**

## NANOPARTICULATE INSULIN FORMULATIONS

Field of the Invention

The present invention is directed to nanoparticulate compositions comprising insulin, having adsorbed to the surface of the insulin particles at least one surface stabilizer.

Background of the InventionA. Background Regarding Nanoparticulate Compositions

Nanoparticulate compositions, first described in U.S. Patent No. 5,145,684 ("the '684 patent"), are particles consisting of a poorly soluble active agent having adsorbed onto the surface thereof a non-crosslinked surface stabilizer. The '684 patent also describes methods of making such nanoparticulate compositions. Nanoparticulate compositions are desirable because with a decrease in particle size, and a consequent increase in surface area, a composition is rapidly dissolved and absorbed following administration. The '684 patent does not teach or suggest nanoparticulate compositions comprising peptides or insulin.

Methods of making nanoparticulate compositions are described, for example, in U.S. Patent Nos. 5,518,187 and 5,862,999, both for "Method of Grinding Pharmaceutical Substances;" U.S. Patent No. 5,718,388, for "Continuous Method of Grinding Pharmaceutical Substances;" and U.S. Patent No. 5,510,118 for "Process of Preparing Therapeutic Compositions Containing Nanoparticles."

Nanoparticulate compositions are also described, for example, in U.S. Patent Nos. 5,298,262 for "Use of Ionic Cloud Point Modifiers to Prevent Particle Aggregation During Sterilization;" 5,302,401 for "Method to Reduce Particle Size Growth During Lyophilization;" 5,318,767 for "X-Ray Contrast Compositions Useful in Medical Imaging;" 5,326,552 for "Novel Formulation For Nanoparticulate X-Ray Blood Pool Contrast Agents Using High Molecular Weight Non-ionic Surfactants;" 5,328,404 for "Method of X-Ray Imaging Using Iodinated Aromatic Propanedioates;" 5,336,507 for "Use of Charged Phospholipids to Reduce Nanoparticle Aggregation;" 5,340,564 for "Formulations Comprising Olin 10-G to Prevent Particle Aggregation and Increase Stability;" 5,346,702 for "Use of Non-Ionic Cloud Point Modifiers to Minimize Nanoparticulate Aggregation During Sterilization;" 5,349,957 for

“Preparation and Magnetic Properties of Very Small Magnetic-Dextran Particles;” 5,352,459 for “Use of Purified Surface Modifiers to Prevent Particle Aggregation During Sterilization;” 5,399,363 and 5,494,683, both for “Surface Modified Anticancer Nanoparticles;” 5,401,492 for “Water Insoluble Non-Magnetic Manganese Particles as Magnetic Resonance Enhancement Agents;” 5,429,824 for “Use of Tyloxapol as a Nanoparticulate Stabilizer;” 5,447,710 for “Method for Making Nanoparticulate X-Ray Blood Pool Contrast Agents Using High Molecular Weight Non-ionic Surfactants;” 5,451,393 for “X-Ray Contrast Compositions Useful in Medical Imaging;” 5,466,440 for “Formulations of Oral Gastrointestinal Diagnostic X-Ray Contrast Agents in Combination with Pharmaceutically Acceptable Clays;” 5,470,583 for “Method of Preparing Nanoparticle Compositions Containing Charged Phospholipids to Reduce Aggregation;” 5,472,683 for “Nanoparticulate Diagnostic Mixed Carbamic Anhydrides as X-Ray Contrast Agents for Blood Pool and Lymphatic System Imaging;” 5,500,204 for “Nanoparticulate Diagnostic Dimers as X-Ray Contrast Agents for Blood Pool and Lymphatic System Imaging;” 5,518,738 for “Nanoparticulate NSAID Formulations;” 5,521,218 for “Nanoparticulate Iododipamide Derivatives for Use as X-Ray Contrast Agents;” 5,525,328 for “Nanoparticulate Diagnostic Diatrizoxy Ester X-Ray Contrast Agents for Blood Pool and Lymphatic System Imaging;” 5,543,133 for “Process of Preparing X-Ray Contrast Compositions Containing Nanoparticles;” 5,552,160 for “Surface Modified NSAID Nanoparticles;” 5,560,931 for “Formulations of Compounds as Nanoparticulate Dispersions in Digestible Oils or Fatty Acids;” 5,565,188 for “Polyalkylene Block Copolymers as Surface Modifiers for Nanoparticles;” 5,569,448 for “Sulfated Non-ionic Block Copolymer Surfactant as Stabilizer Coatings for Nanoparticle Compositions;” 5,571,536 for “Formulations of Compounds as Nanoparticulate Dispersions in Digestible Oils or Fatty Acids;” 5,573,749 for “Nanoparticulate Diagnostic Mixed Carboxylic Anydrides as X-Ray Contrast Agents for Blood Pool and Lymphatic System Imaging;” 5,573,750 for “Diagnostic Imaging X-Ray Contrast Agents;” 5,573,783 for “Redispersible Nanoparticulate Film Matrices With Protective Overcoats;” 5,580,579 for “Site-specific Adhesion Within the GI Tract Using Nanoparticles Stabilized by High Molecular Weight, Linear Poly(ethylene Oxide) Polymers;” 5,585,108 for “Formulations of Oral Gastrointestinal Therapeutic Agents in Combination with Pharmaceutically Acceptable Clays;” 5,587,143 for “Butylene Oxide-Ethylene Oxide Block Copolymers Surfactants as Stabilizer Coatings for Nanoparticulate Compositions;” 5,591,456 for “Milled Naproxen with

Hydroxypropyl Cellulose as Dispersion Stabilizer;" 5,593,657 for "Novel Barium Salt Formulations Stabilized by Non-ionic and Anionic Stabilizers;" 5,622,938 for "Sugar Based Surfactant for Nanocrystals;" 5,628,981 for "Improved Formulations of Oral Gastrointestinal Diagnostic X-Ray Contrast Agents and Oral Gastrointestinal Therapeutic Agents;" 5,643,552 for "Nanoparticulate Diagnostic Mixed Carbonic Anhydrides as X-Ray Contrast Agents for Blood Pool and Lymphatic System Imaging;" 5,718,388 for "Continuous Method of Grinding Pharmaceutical Substances;" 5,718,919 for "Nanoparticles Containing the R(-)Enantiomer of Ibuprofen;" 5,747,001 for "Aerosols Containing Beclomethasone Nanoparticle Dispersions;" 5,834,025 for "Reduction of Intravenously Administered Nanoparticulate Formulation Induced Adverse Physiological Reactions;" 6,045,829 "Nanocrystalline Formulations of Human Immunodeficiency Virus (HIV) Protease Inhibitors Using Cellulosic Surface Stabilizers;" 6,068,858 for "Methods of Making Nanocrystalline Formulations of Human Immunodeficiency Virus (HIV) Protease Inhibitors Using Cellulosic Surface Stabilizers;" 6,153,225 for "Injectable Formulations of Nanoparticulate Naproxen;" 6,165,506 for "New Solid Dose Form of Nanoparticulate Naproxen;" 6,221,400 for "Methods of Treating Mammals Using Nanocrystalline Formulations of Human Immunodeficiency Virus (HIV) Protease Inhibitors;" 6,264,922 for "Nebulized Aerosols Containing Nanoparticle Dispersions;" 6,267,989 for "Methods for Preventing Crystal Growth and Particle Aggregation in Nanoparticle Compositions;" 6,270,806 for "Use of PEG-Derivatized Lipids as Surface Stabilizers for Nanoparticulate Compositions;" 6,316,029 for "Rapidly Disintegrating Solid Oral Dosage Form;" 6,375,986 for "Solid Dose Nanoparticulate Compositions Comprising a Synergistic Combination of a Polymeric Surface Stabilizer and Dioctyl Sodium Sulfosuccinate;" 6,428,814 for "Bioadhesive Nanoparticulate Compositions Having Cationic Surface Stabilizers;" and 6,432,381 for "Methods for Targeting Drug Delivery to the Upper and/or Lower Gastrointestinal Tract," all of which are specifically incorporated by reference. In addition, U.S. Patent Application No. 20020012675 A1, published on January 31, 2002, for "Controlled Release Nanoparticulate Compositions," describes nanoparticulate compositions, and is specifically incorporated by reference.

Nanoparticulate peptides and proteins are referenced in U.S. Patent No. 6,375,986 for "Solid Dose Nanoparticulate Compositions Comprising a Synergistic Combination of a Polymeric Surface Stabilizer and Dioctyl Sodium Sulfosuccinate," U.S. Patent No. 6,428,814 for

"Bioadhesive Nanoparticulate Compositions Having Cationic Surface Stabilizers," U.S. Patent Application No. 20020012675 A1 for "Controlled Release Nanoparticulate Compositions," WO 00/51572 for "Use of PEG-Derivatized Lipids as Surface Stabilizers for Nanoparticle Compositions," published on September 8, 2000, and WO 00/53164 for "Methods for Preventing Crystal Growth and Particle Aggregation in Nanoparticulate Compositions, published on September 14, 2000. However, none of these references teach or suggest that the peptide or protein can be insulin.

Amorphous small particle compositions are described, for example, in U.S. Patent Nos. 4,783,484 for "Particulate Composition and Use Thereof as Antimicrobial Agent;" 4,826,689 for "Method for Making Uniformly Sized Particles from Water-Insoluble Organic Compounds;" 4,997,454 for "Method for Making Uniformly-Sized Particles From Insoluble Compounds;" 5,741,522 for "Ultrasmall, Non-aggregated Porous Particles of Uniform Size for Entrapping Gas Bubbles Within and Methods;" and 5,776,496, for "Ultrasmall Porous Particles for Enhancing Ultrasound Back Scatter." None of these references relates to a nanoparticulate insulin composition.

#### B. Background Regarding Delivery of Peptides and Proteins

Delivery of peptides and proteins has long been a problem because of high toxicity, poor bioavailability, and degrading of the peptide or protein when formulated for administration or application. Numerous approaches to formulating peptide and protein compositions have been developed. For example, U.S. Patent No. 5,889,110 discloses microparticles comprising a salt formed from a cation derived from a peptide containing one or more basic groups and an anion derived from a carboxy-terminated polyester. The composition is prepared from a stoichiometric equivalent of the polyester carboxylic acid end groups relative to the basic peptide groups, obtainable by a complicated and expensive process.

Such a process comprises: (i) dissolving the basic peptide and carboxy-terminated polyester in a first solvent in which both the peptide and the polyester are soluble to form a first solution; (ii) freezing the first solution at high speed to form a frozen mixture; (iii) freeze-drying the frozen mixture to remove the first solvent to form a freeze-dried product; (iv) dispersing the freeze-dried product into a second solvent which is a solvent for the polyester and a non-solvent for the peptide to form a second solution containing the peptide/polyester salt; and (v) removing

the second solvent from the second solution by spray-drying, spray-congealing, evaporation, or phase separation coacervation to form a microparticulate solid product.

U.S. Patent No. 5,354,562 discloses a process for preparing micronized polypeptide drugs in a powder form suitable for aerosol administration or for use in injectable suspensions by lyophilization followed by jet milling. U.S. Patent No. 6,051,694 discloses a method of size reduction of proteins comprising contacting the solid protein with a critical fluid in which the protein is essentially insoluble, followed by depressurization of the protein and critical fluid mixture.

None of the prior art methods provide nanoparticulate insulin compositions. In fact, attempts to mill peptides such as insulin have resulted in a change of conformation of the peptides. For example, Leung et al., *J. Pharm. Sci.*, 87:501-7 (1998), disclose transformation of the crystal structure of aspartame hemihydrate into a different polymorph upon ball milling of the peptide. This is not surprising, as peptides and proteins can be labile to milling conditions, e.g., shear, pressure, and temperature.

There remains a need in the art for nanoparticulate insulin formulations that would facilitate insulin delivery in high dosages with consistent and effective activity and other advantages in comparison to any conventional preparations presently available. In accordance with the present invention, such a preparation of insulin is provided.

### Summary of the Invention

The present invention is directed to the surprising and unexpected discovery that stable nanoparticulate compositions of insulin can be made. The nanoparticulate compositions, which comprise insulin and at least one surface stabilizer adsorbed to the surface thereof, are advantageous in comparison to prior art insulin preparations in that they possess both a rapid onset of activity and prolonged activity.

The invention is further directed to methods of making nanoparticle insulin compositions and pharmaceutical dosage forms containing them. The method of preparing the insulin compositions comprises contacting insulin, e.g. insoluble bovine insulin or recombinant insulin, with at least one surface stabilizer for a time and under conditions sufficient to provide a stable nanoparticulate composition. The particle size of insulin is reduced by milling or

homogenization. The one or more surface stabilizers can be contacted with the insulin either before, during, or after size reduction thereof.

The present invention is further directed to treatment of conditions requiring insulin therapy.

#### Brief Description of the Figures

- FIG 1: Are scanning electron microscope images at 5,000 magnification of insulin particles before and after milling in accordance with the method of the present invention;
- FIG 2: Shows the results of a reducing SDS PAGE gel of the supernatant and pellet of a nanoparticulate insulin sample in two concentrations to determine whether milling caused a loss of insulin;
- FIG 3: Shows the results of a reducing SDS PAGE gel of the pellet of a nanoparticulate insulin sample in two concentrations to determine whether milling caused a loss of insulin;
- FIG 4: Shows the pharmacokinetic parameters of insulin resulting from injection of nanoparticulate insulin samples via intramuscular, subcutaneous and intraperitoneal routes in comparison to a control insulin solution:
- FIG 5: Is a bar graph illustrating the AUC ( $\mu\text{U/ml.h}$ ) of insulin from injection of nanoparticulate insulin samples via subcutaneous, intramuscular and intraperitoneal routes in comparison to a control insulin solution;
- FIG. 6: Is a plot showing blood insulin concentration over time following injection of nanoparticulate insulin samples via subcutaneous, intramuscular and intraperitoneal routes in comparison to a reference insulin solution;
- FIG. 7: Is a plot showing blood glucose concentration over time following injection of nanoparticulate insulin samples via subcutaneous, intramuscular and intraperitoneal routes in comparison to a reference insulin solution; and
- FIG. 8 Is a plot showing the change in blood glucose concentration over time following injection of nanoparticulate insulin samples via subcutaneous, intramuscular and intraperitoneal routes in comparison to a reference insulin solution.



### Detailed Description of the Invention

The present invention is directed to compositions of nanoparticulate insulin and methods for their preparation and use in insulin therapy. Prior to the present invention, it was known that crystalline drugs could be formulated into nanoparticulate compositions by the method taught in the '684 patent. However, as discussed above, formulations of nanoparticulate insulin were not contemplated, partly due to the difficulty in retaining the insulin conformational structure upon milling to a particle size in the nanometer region. This is significant, as a change in conformation of insulin can correlate with a loss of activity.

Nanoparticulate insulin, prepared in accordance to the present invention, comprises at least one surface stabilizer that is adsorbed to the insulin particles. The surface stabilizer acts as a steric barrier to other insulin particles thereby preventing agglomeration and particle size growth. This results in a stable nanoparticulate composition, in which the particle size of the composition does not significantly increase over time via solubilization and recrystallization or agglomeration. Individually adsorbed molecules of the surface stabilizer are essentially free of intermolecular crosslinkages.

Insulin is a 5.8 kD protein hormone important in regulation of fuel metabolism. Secretion of insulin by the  $\beta$  cells of the pancreas is stimulated by glucose and the parasympathetic nervous system. Insulin promotes the dephosphorylation of key interconvertible enzymes. One consequence is to stimulate glycogen synthesis in both muscle and liver and to suppress gluconeogenesis by the liver. Insulin also accelerates glycolysis in the liver, which in turn increases the synthesis of fatty acids. The entry of glucose into muscle and adipose cells is promoted by insulin. The abundance of fatty acids and glucose in adipose tissue results in the synthesis and storage of triacylglycerols. The action of insulin also extends to amino acid and protein metabolism. Insulin promotes the uptake of branched-chain amino acids by muscle, which favors a building up of muscle protein. In sum, insulin has a general stimulating effect on protein synthesis.

Three different compositions of insulin are available: soluble, insoluble, and a mixture thereof. The difference among these compositions is in the observed bioavailability at physiological conditions. Insoluble insulin, and a mixture of soluble and insoluble insulin, have a slow onset of activity, about 2 hours, but a prolonged duration that extends for up to about 24 and 13-14 hours, respectively. Soluble insulin has a more rapid onset of activity, about 30

minutes, but with only about six hours duration of activity. The nanoparticle insulin of the present invention is superior to prior art insulin compositions in that it displays a rapid onset of activity, such as that found with soluble insulin, and it exhibits a prolonged duration of activity, such as that found in insoluble and soluble-insoluble insulin mixtures.

The insulin composition can be formulated for administration via, for example, oral, pulmonary, nasal, parenteral, rectal, local, buccal, or topical administration.

The insulin particles can be in the form of crystalline particles, semi-crystalline particles, semi-amorphous particles, amorphous particles, or a mixture thereof.

The concentration of insulin in the compositions of the invention can vary from about 99.5% to about 0.001%, from about 95% to about 0.1%, or from about 90% to about 0.5%, by weight, based on the total combined dry weight of the insulin and at least one surface stabilizer, not including other excipients.

#### A. Useful Surface Stabilizers

Suitable surface stabilizers utilized in preparing the subject nanoparticle insulin are preferably selected from known organic and inorganic pharmaceutical excipients. Two or more surface stabilizers may be used in combination. The surface stabilizers protect insulin from degradation and potential protease cleavage in addition to preventing agglomeration and particle size growth as described above.

Such organic and inorganic pharmaceutical excipients include various polymers, low molecular weight oligomers, natural products, and surfactants. Preferred surface stabilizers include nonionic and ionic surfactants (*e.g.*, cationic and anionic surfactants). Representative examples of surface stabilizers include cetyl pyridinium chloride, gelatin, lecithin (phosphatides), dextran, glycerol, cholesterol, tragacanth, stearic acid, its salts and its esters, cetomacrogol emulsifying wax, sorbitan esters, polyoxyethylene alkyl ethers, polyoxyethylene castor oil derivatives, polyoxyethylene sorbitan fatty acid esters, *e.g.*, the commercially available Tweens® such as Tween 80® (ICI Specialty Chemicals); polyethylene glycols, *e.g.*, Carbowaxs 3350® and 1450®, and Carbopol 934® (Union Carbide), dodecyl trimethyl ammonium bromide, polyoxyethylene stearates, colloidal silicon dioxide, sodium dodecylsulfate, carboxymethylcellulose calcium, hydroxypropyl celluloses (*e.g.*, HPC, HPC-SL, and HPC-L), hydroxypropyl methylcellulose (HPMC), carboxymethylcellulose sodium, methylcellulose,

hydroxyethylcellulose, hydroxypropylmethyl-cellulose phthalate, noncrystalline cellulose, magnesium aluminum silicate, triethanolamine, polyvinyl alcohol (PVA), polyvinylpyrrolidone (PVP), 4-(1,1,3,3-tetramethylbutyl)-phenol polymer with ethylene oxide and formaldehyde (also known as tyloxapol), poloxamers, *e.g.*, Pluronic F68® and F108®, which are block copolymers of ethylene oxide and propylene oxide; poloxamines, *e.g.*, Tetronic 908®, also known as Poloxamine 908®, which is a tetrafunctional block copolymer derived from sequential addition of propylene oxide and ethylene oxide to ethylenediamine (BASF Corporation, Parsippany, N.J.); a charged phospholipid such as dimyristoyl phosphatidyl glycerol, dioctylsulfosuccinate (DOSS); Tetronic 1508® (T-1508) (BASF Corporation), dialkylesters of sodium sulfosuccinic acid (*e.g.*, Aerosol OT®, which is a dioctyl ester of sodium sulfosuccinic acid (American Cyanamid)); sodium lauryl sulfate (SLS), such as Duponol P® (DuPont); Tritons X-200®, which is an alkyl aryl polyether sulfonate (Rohm and Haas); Crodestas F-110®, which is a mixture of sucrose stearate and sucrose distearate (Croda Inc.); p-isononylphenoxypoly-(glycidol), also known as Olin-LOG® or Surfactant 10-G® (Olin Chemicals, Stamford, CT); Crodestas SL-40® (Croda, Inc.); poly (2-methacryloxyethyltrimethylammonium bromide) (S1001); poly(N-vinylpyrrolidone/2-dimethylaminoethyl methacrylate) di methylsulphate quaternary (S1002); poly(2-methylacryloxyamidopropyltrimethylammonium chloride)(S1004); sodium deoxycholic acid; vitamin E derivatized with polyethylene glycol (PEG), a phospholipid derivatized with PEG, cholesterol derivatized with PEG, a cholesterol derivative derivatized with PEG, vitamin A derivatized with PEG, benzalkonium chloride (BKC), centrimide (a quaternary ammonium compound), Span® 20 (sorbitan monolaurate), Plasdone® S630, (a random copolymer of polyvinylpyrrolidone (PVP) and vinyl acetate in a 60:40 ratio), hexyldecyltrimethylammonium bromide (HDMAB), triblock copolymers of the structure -(-PEO)--(-PBO)--(-PEO)- (known as B20-5000), poly (2-methacryloxyethyltrimethylammonium bromide) (S1001), poly(N-vinylpyrrolidone/2-dimethylaminoethyl methacrylate) di methylsulphate quaternary (S1002), poly(2-methylacryloxyamidopropyltrimethylammonium chloride) (S1004), random copolymers of PVP and vinyl acetate, lysozyme, and the like.

Examples of useful cationic surface stabilizers include, but are not limited to, polymers, biopolymers, polysaccharides, cellulosics, alginates, phospholipids, and nonpolymeric compounds, such as zwitterionic stabilizers, poly-n-methylpyridinium, anthryl pyridinium

chloride, cationic phospholipids, chitosan, polylysine, polyvinylimidazole, polybrene, polymethylmethacrylate trimethylammoniumbromide bromide (PMMTMABr), hexyldesyltrimethylammonium bromide (HDMAB), and polyvinylpyrrolidone-2-dimethylaminoethyl methacrylate dimethyl sulfate.

Other useful cationic stabilizers include, but are not limited to, cationic lipids, sulfonium, phosphonium, and quarternary ammonium compounds, such as stearyltrimethylammonium chloride, benzyl-di(2-chloroethyl)ethylammonium bromide, coconut trimethyl ammonium chloride or bromide, coconut methyl dihydroxyethyl ammonium chloride or bromide, decyl triethyl ammonium chloride, decyl dimethyl hydroxyethyl ammonium chloride or bromide, C<sub>12</sub>-<sub>15</sub>dimethyl hydroxyethyl ammonium chloride or bromide, coconut dimethyl hydroxyethyl ammonium chloride or bromide, myristyl trimethyl ammonium methyl sulphate, lauryl dimethyl benzyl ammonium chloride or bromide, lauryl dimethyl (ethenoxy)<sub>4</sub> ammonium chloride or bromide, N-alkyl (C<sub>12-18</sub>)dimethylbenzyl ammonium chloride, N-alkyl (C<sub>14-18</sub>)dimethyl-benzyl ammonium chloride, N-tetradecyldimethylbenzyl ammonium chloride monohydrate, dimethyl didecyl ammonium chloride, N-alkyl and (C<sub>12-14</sub>) dimethyl 1-naphthylmethyl ammonium chloride, trimethylammonium halide, alkyl-trimethylammonium salts and dialkyl-dimethylammonium salts, lauryl trimethyl ammonium chloride, ethoxylated alkyamidoalkyldialkylammonium salt and/or an ethoxylated trialkyl ammonium salt, dialkylbenzene dialkylammonium chloride, N-didecyl dimethyl ammonium chloride, N-tetradecyldimethylbenzyl ammonium, chloride monohydrate, N-alkyl(C<sub>12-14</sub>) dimethyl 1-naphthylmethyl ammonium chloride and dodecyl dimethylbenzyl ammonium chloride, dialkyl benzenealkyl ammonium chloride, lauryl trimethyl ammonium chloride, alkylbenzyl methyl ammonium chloride, alkyl benzyl dimethyl ammonium bromide, C<sub>12</sub>, C<sub>15</sub>, C<sub>17</sub> trimethyl ammonium bromides, dodecylbenzyl triethyl ammonium chloride, poly-diallyldimethylammonium chloride (DADMAC), dimethyl ammonium chlorides, alkyl dimethylammonium halogenides, tricetyl methyl ammonium chloride, decyltrimethylammonium bromide, dodecyltriethylammonium bromide, tetradecyltrimethylammonium bromide, methyl trioctylammonium chloride (ALQUAT 336™), POLYQUAT 10™, tetrabutylammonium bromide, benzyl trimethylammonium bromide, choline esters (such as choline esters of fatty acids), benzalkonium chloride, stearylkonium chloride compounds (such as stearyltrimonium chloride and Di-stearyldimonium chloride), cetyl pyridinium bromide or chloride, halide salts of quaternized polyoxyethylalkylamines,

MIRAPOL™ and ALKAQUAT™ (Alkaril Chemical Company), alkyl pyridinium salts; amines, such as alkylamines, dialkylamines, alkanolamines, polyethylenepolyamines, N,N-dialkylaminoalkyl acrylates, and vinyl pyridine, amine salts, such as lauryl amine acetate, stearyl amine acetate, alkylpyridinium salt, and alkylimidazolium salt, and amine oxides; imide azolinium salts; protonated quaternary acrylamides; methylated quaternary polymers, such as poly[diallyl dimethylammonium chloride] and poly-[N-methyl vinyl pyridinium chloride]; and cationic guar.

Such exemplary cationic surface stabilizers and other useful cationic surface stabilizers are described in J. Cross and E. Singer, *Cationic Surfactants: Analytical and Biological Evaluation* (Marcel Dekker, 1994); P. and D. Rubingh (Editor), *Cationic Surfactants: Physical Chemistry* (Marcel Dekker, 1991); and J. Richmond, *Cationic Surfactants: Organic Chemistry*, (Marcel Dekker, 1990).

Particularly preferred nonpolymeric primary stabilizers are any nonpolymeric compound, such as benzalkonium chloride, a carbonium compound, a phosphonium compound, an oxonium compound, a halonium compound, a cationic organometallic compound, a quaternary phosphorous compound, a pyridinium compound, an anilinium compound, an ammonium compound, a hydroxylammonium compound, a primary ammonium compound, a secondary ammonium compound, a tertiary ammonium compound, and quaternary ammonium compounds of the formula  $NR_1R_2R_3R_4^{(+)}$ . For compounds of the formula  $NR_1R_2R_3R_4^{(+)}$ :

- (i) none of  $R_1$ - $R_4$  are  $CH_3$ ;
- (ii) one of  $R_1$ - $R_4$  is  $CH_3$ ;
- (iii) three of  $R_1$ - $R_4$  are  $CH_3$ ;
- (iv) all of  $R_1$ - $R_4$  are  $CH_3$ ;
- (v) two of  $R_1$ - $R_4$  are  $CH_3$ , one of  $R_1$ - $R_4$  is  $C_6H_5CH_2$ , and one of  $R_1$ - $R_4$  is an alkyl chain of seven carbon atoms or less;
- (vi) two of  $R_1$ - $R_4$  are  $CH_3$ , one of  $R_1$ - $R_4$  is  $C_6H_5CH_2$ , and one of  $R_1$ - $R_4$  is an alkyl chain of nineteen carbon atoms or more;
- (vii) two of  $R_1$ - $R_4$  are  $CH_3$  and one of  $R_1$ - $R_4$  is the group  $C_6H_5(CH_2)_n$ , where  $n > 1$ ;
- (viii) two of  $R_1$ - $R_4$  are  $CH_3$ , one of  $R_1$ - $R_4$  is  $C_6H_5CH_2$ , and one of  $R_1$ - $R_4$  comprises at least one heteroatom;

- (ix) two of  $R_1$ - $R_4$  are  $CH_3$ , one of  $R_1$ - $R_4$  is  $C_6H_5CH_2$ , and one of  $R_1$ - $R_4$  comprises at least one halogen;
- (x) two of  $R_1$ - $R_4$  are  $CH_3$ , one of  $R_1$ - $R_4$  is  $C_6H_5CH_2$ , and one of  $R_1$ - $R_4$  comprises at least one cyclic fragment;
- (xi) two of  $R_1$ - $R_4$  are  $CH_3$  and one of  $R_1$ - $R_4$  is a phenyl ring; or
- (xii) two of  $R_1$ - $R_4$  are  $CH_3$  and two of  $R_1$ - $R_4$  are purely aliphatic fragments.

Such compounds include, but are not limited to, behenalkonium chloride, benzethonium chloride, cetylpyridinium chloride, behentrimonium chloride, lauralkonium chloride, cetalkonium chloride, cetrimonium bromide, cetrimonium chloride, cethylamine hydrofluoride, chlorallylmethenamine chloride (Quaternium-15), distearyldimonium chloride (Quaternium-5), dodecyl dimethyl ethylbenzyl ammonium chloride (Quaternium-14), Quaternium-22, Quaternium-26, Quaternium-18 hectorite, dimethylaminoethylchloride hydrochloride, cysteine hydrochloride, diethanolammonium POE (10) oleyl ether phosphate, diethanolammonium POE (3) oleyl ether phosphate, tallow alkonium chloride, dimethyl dioctadecylammonium bentonite, stearylalkonium chloride, domiphen bromide, denatonium benzoate, myristalkonium chloride, laurtrimonium chloride, ethylenediamine dihydrochloride, guanidine hydrochloride, pyridoxine HCl, iofetamine hydrochloride, meglumine hydrochloride, methylbenzethonium chloride, myrtrimonium bromide, oleyltrimonium chloride, polyquaternium-1, procaine hydrochloride, cocobetaine, stearylalkonium bentonite, stearylalkonium hectorite, stearyl trihydroxyethyl propylenediamine dihydrofluoride, tallowtrimonium chloride, and hexadecyltrimethyl ammonium bromide.

Most of these surface stabilizers are known pharmaceutical excipients and are described in detail in the Handbook of Pharmaceutical Excipients, published jointly by the American Pharmaceutical Association and The Pharmaceutical Society of Great Britain (The Pharmaceutical Press, 2000), specifically incorporated by reference. The surface stabilizers are commercially available and/or can be prepared by techniques known in the art starting from known materials.

The concentration of the at least one surface stabilizer can vary from about 0.001 to about 99.5%, from about 0.1% to about 95%, and from about 0.5% to about 90%, by weight, based on

the total combined dry weight of the insulin and at least one surface stabilizer, not including other excipients.

**B. Particle Size of the Insulin Particles**

Preferably, the insulin nanoparticles of the invention have an effective average particle size of less than about 5 microns, less than about 4 microns, less than about 3 microns, less than about 2 microns, less than about 1500 nm, less than about 1 micron, less than about 800 nm, less than about 700 nm, less than about 600 nm, less than about 500 nm, less than about 400 nm, less than about 300 nm, less than about 200 nm, less than about 100 nm, less than about 75 nm, or less than about 50 nm, as measured by light-scattering methods or other methods accepted in the art. By "an effective average particle size of less than about 5 microns" it is meant that at least about 50% of the insulin particles have a weight average particle size of less than about 5 microns when measured by light scattering or other conventional techniques. In addition, in other embodiments of the invention, at least about 70%, about 90%, or about 95% of the insulin particles have an effective average particle size of less than about 5 microns, less than about 4 microns, less than about 3 microns, less than about 2 microns, less than about 1500 nm, less than about 1 micron, less than about 800 nm, less than about 700 nm, less than about 600 nm, less than about 500 nm, less than about 400 nm, less than about 300 nm, less than about 200 nm, less than about 100 nm, less than about 75 nm, or less than about 50 nm.

**C. Methods of Making Nanoparticulate Insulin Compositions**

Nanoparticulate active agent compositions can be made using, for example, milling, or homogenization techniques. Exemplary methods of making nanoparticulate compositions are described in U.S. Patent No. 5,145,684. Methods of making nanoparticulate compositions are also described in U.S. Patent No. 5,518,187 for "Method of Grinding Pharmaceutical Substances;" U.S. Patent No. 5,718,388 for "Continuous Method of Grinding Pharmaceutical Substances;" U.S. Patent No. 5,862,999 for "Method of Grinding Pharmaceutical Substances;" U.S. Patent No. 5,543,133 for "Process of Preparing X-Ray Contrast Compositions Containing Nanoparticles;" U.S. Patent No. 5,534,270 for "Method of Preparing Stable Drug Nanoparticles;" U.S. Patent No. 5,510,118 for "Process of Preparing Therapeutic Compositions Containing Nanoparticles;" and U.S. Patent No. 5,470,583 for "Method of Preparing

Nanoparticle Compositions Containing Charged Phospholipids to Reduce Aggregation,” all of which are specifically incorporated by reference.

The optimal effective average particle size of the invention can be obtained by controlling the process of particle size reduction, such as by controlling the milling time and the amount of surface stabilizer added. Particle size growth and particle aggregation can also be minimized by milling and/or storing the composition under colder temperatures.

The nanoparticulate insulin dispersions made via, for example, milling or homogenization can be utilized in solid, semi-solid, or liquid dosage formulations, such as controlled release formulations, solid dose fast melt formulations, aerosol formulations, lyophilized formulations, tablets, solid lozenge, capsules, powders, liquids for injection, etc.

#### 1. Milling to Obtain Nanoparticulate Insulin Compositions

Milling insulin to obtain a nanoparticulate composition comprises dispersing insulin particles in a liquid dispersion medium in which insulin is poorly soluble, followed by applying mechanical means in the presence of grinding media to reduce the particle size of the insulin to the desired effective average particle size. The dispersion medium can be, for example, water, safflower oil, ethanol, t-butanol, glycerin, polyethylene glycol (PEG), hexane, or glycol.

The insulin particles can be reduced in size in the presence of at least one surface stabilizer. Alternatively, the insulin particles can be contacted with one or more surface stabilizers after attrition. It is preferred, however, to disperse the insulin in the liquid dispersion medium in the presence of the at least one surface stabilizer as an aid in wetting of the insulin particles. Other compounds, such as a diluent, can be added to the insulin/surface stabilizer composition during the size reduction process. Dispersions can be manufactured continuously or in a batch mode.

#### 2. Homogenization to Obtain Insulin Nanoparticulate Compositions

Exemplary homogenization methods of preparing active agent nanoparticulate compositions are described in U.S. Patent No. 5,510,118, for “Process of Preparing Therapeutic Compositions Containing Nanoparticles.”

Such a method comprises dispersing insulin particles in a liquid dispersion medium, followed by subjecting the dispersion to homogenization to reduce the particle size of the insulin



to the desired effective average particle size. The insulin particles can be reduced in size in the presence of at least one surface stabilizer. Alternatively, the insulin particles can be contacted with one or more surface stabilizers either before or after particle size reduction. It is preferred, however, to disperse the insulin in the liquid dispersion medium in the presence of the at least one surface stabilizer as an aid to wetting of the insulin particles. Other compounds, such as a diluent, can be added to the insulin/surface stabilizer composition either before, during, or after the size reduction process. Dispersions can be manufactured continuously or in a batch mode.

#### D. Excipients

Pharmaceutical compositions in accordance with the present invention include nanoparticulate insulin compositions formulated together with one or more non-toxic, physiologically acceptable carriers, adjuvants, or vehicles, collectively referred to as carriers. Such carriers may comprise physiologically acceptable sterile aqueous or nonaqueous solutions, dispersions, suspensions or emulsions, and sterile powders for reconstitution into such preparations. Examples of suitable aqueous and nonaqueous carriers, diluents, solvents, or vehicles include water, ethanol, polyols (propyleneglycol, polyethyleneglycol, glycerol, and the like), suitable mixtures thereof, vegetable oils (such as olive oil) and injectable organic esters such as ethyl oleate. Proper fluidity can be maintained, for example, by the use of a coating such as lecithin, by the maintenance of the required particle size in the case of dispersions and by the use of surfactants.

The nanoparticulate compositions may also contain adjuvants such as preserving, wetting, emulsifying, and dispensing agents. Prevention of the growth of microorganisms can be ensured by various antibacterial and antifungal agents, such as potassium sorbate, methylparaben, propylparaben, benzoic acid and its salts, other esters of parahydroxybenzoic acid such as butylparaben, alcohols such as ethyl or benzyl alcohol, phenolic compounds such as phenol, or quaternary compounds such as benzalkonium chloride, parabens, chlorobutanol, phenol, sorbic acid, and the like. It may also be desirable to include isotonic agents, such as sugars, sodium chloride, and the like. Prolonged absorption of the injectable pharmaceutical form can be brought about by the use of agents delaying absorption, such as aluminum monostearate and gelatin.

In one embodiment of the invention, the nanoparticulate compositions of the invention are intended for parenteral administration to humans and animals by known routes of parenteral administration, *i.e.*, intravenous, intramuscular, subcutaneous, or intraperitoneal. Other dosage forms can also be utilized, such as aerosols, tablets, etc.

Actual dosage levels of the nanoparticulate insulin compositions of the invention are given as standardized units of insulin as is the case with conventional insulin preparations and the dosage administered will depend on recognized factors such as the time of administration and the body weight, general health, age, sex, and diet of the patient.

The following examples are given to illustrate the present invention. It should be understood, however, that the invention is not to be limited to the specific conditions or details described in these examples. Throughout the specification, all references to publicly available documents, including U.S. patents, are specifically incorporated herein by reference.

#### Example 1

The purpose of this example was to prepare nanoparticulate insulin formulations using low energy milling techniques.

Insulin and one or more surface stabilizers, in the amounts shown in Table 1, were mixed with water to form a pre-milling slurry. This slurry was then added to a sealable vessel and rotated for from 1 to 4 days at a pre-set rotational speed (typically 100-200 rpm) on a roller mill using high wear zirconia grinding media (Tosoh Corporation, Tokyo, Japan) with a diameter of 0.8 mm. This low energy milling technique relies upon gravitational grinding mechanisms to break the particle size down, hence the use of heavy ceramic media.

Particle size distributions of the resultant insulin compositions were determined using a Horiba LA-910 light-scattering particle size analyzer (Horiba Instruments, Irvine, CA). The results shown in Table 1 were determined at 24 hours post milling. It is to be noted that the concentration of insulin in this Example was 5%, but could have been, for example, 1%, 2%, 3%, or 10% or higher.

The surface stabilizers used include: (1) poly (2-methacryloxyethyl-trimethylammonium bromide) (S1001); (2) poly(N-vinylpyrrolidone/2-dimethyl-aminoethyl methacrylate) dimethylsulphate quarternary (S1002); (3) poly(2-methylacryloxyamido-propyltrimethylammonium chloride) (S1004); (4) Pluronic® F68; (5) hydroxypropyl cellulose

(HPC); (6) sodium deoxycholic acid; (7) vitamin E derivatized with polyethylene glycol (PEG); and (8) Tween® 80.

| <b>TABLE 1</b><br><b>Low Energy Ball-Milled Nanoparticulate Insulin Formulations</b><br><b>(Particle Size 24 hrs after Milling)</b> |              |  |  |  |
|---|--------------|--|--|--|
| Exp No.   | % Drug       | % of Stabilizer                            | Mean Particle Size @ 24 hours after milling (nm) | Particle size of 90% @ 24 hours after milling (nm) |
| 1   | 5.0% insulin | 0.0  | 4198<br>(1344 after 1 min sonication)            | 7092<br>(3104 after 1 min sonication)              |
| 2   | 5.0% insulin | 1% F68 0.1% Sodium Deoxycholic Acid        | 165<br>(144 after 1 min sonication)              | 266<br>(193 after 1 min sonication)                |
| 3   | 5.0% insulin | 1% PVP k17 0.1% Sodium Deoxycholic Acid    | 199<br>(163 after 1 min sonication)              | 279<br>(219 after 1 min sonication)                |
| 4   | 5.0% insulin | 1% PVP k29-32 0.1% Sodium Deoxycholic Acid | 243<br>(171 after 1 min sonication)              | 364<br>(250 after 1 min sonication)                |
| 5   | 5.0% insulin | 1% S1004                                   | 252  | 362  |
| 6   | 5.0% insulin | 1% S1001                                   | 282<br>(258 after 1 min sonication)              | 401<br>(380 after 1 min sonication)                |

The measurements reported in Table 2 were taken at one week post milling to determine stability for a representative group of the nanoparticulate compositions shown in Table 1. In several instances, the particle size of the composition was smaller after one week than at 24 hours post milling. While not being bound to any one theory, it is believed that the stabilizer and drug particle may go through a thermodynamic relaxation period. Another theory is that in conjunction with relaxation or separately therefrom, there are interactions between the insulin particle and the stabilizer. Further, a phenomenon may be occurring at the surface of the insulin particle that is similar to the use of cationic stabilizers to complex and condense DNA in solution, *i.e.* the stabilizer interaction may cause a slight condensation on the surface of the insulin particle, resulting in the observed reduction in particle size.

**TABLE 2**  
**Low Energy Ball-Milled Nanoparticulate Insulin Formulations**  
**(Particle Size 24 hrs and 1 Week After Milling)**

| Exp No. | % Drug       | % of Stabilizer                               | Mean Particle Size @ 24 hours after milling (nm) | Particle size of 90% @ 24 hours after milling (nm) | Mean Particle Size @ 1 week after milling (nm) | Particle size of 90% @ 1 week after milling (nm) |
|---------|--------------|---|--|--|--|--|
| 4       | 5.0% insulin | 1% PVP k29-32<br>0.1% Sodium Deoxycholic Acid | 243<br>(171 after 1 min sonication)              | 364<br>(250 after 1 min sonication)                | 212<br>(165 after 1 min sonication)            | 301<br>(222 after 1 min sonication)              |
| 5       | 5.0% insulin | 1% S1004                                      | 252  | 362  | 263  | 364  |
| 6       | 5.0% insulin | 1% S1001                                      | 282<br>(258 after 1 min sonication)              | 401<br>(380 after 1 min sonication)                | 303<br>(266 after 1 min sonication)            | 450<br>(384 after 1 min sonication)              |
| 7       | 5.0% insulin | 1% S1001                                      | 521  | 1004   | 498  | 905  |

### Example 2

The purpose of this example was to prepare nanoparticulate insulin formulations using high energy milling conditions.

Samples of insulin were prepared using a high energy attrition media mill, NanoMill™ (Elan Drug Delivery, King of Prussia, PA). A high energy mill is designed to apply a much higher rotational velocity to the particulate dispersion (100-6000 rpm, typically 5000 rpm), than that used in low energy milling processes. The elevated rotational velocity imparts very high shear conditions within the milling chamber. It is this shear force which causes the particle size reduction.

The media used in this milling technology is a much lighter, highly crosslinked polystyrene media. For preparation of nanoparticulate insulin compositions described in Table 3, 500  $\mu$ m media was used. Other media sizes that could be used range from 50  $\mu$ m to 500  $\mu$ m. The samples were milled from about 30 minutes up to about 3 hours. Particle size distributions were determined using a Horiba LA-910 light-scattering particle size analyzer (Horiba

Instruments, Irvine, CA). Particle size was determined for all three compositions following milling, for Sample 2 at 24 hours following milling, and for Samples 1 and 2 at 1 week after milling. The results are shown in Table 3.

| <b>Exp. No.</b> | <b>% of Insulin</b> | <b>% of Stabilizer</b> | <b>Mean Particle Size (nm) @ harvest</b> | <b>Particle Size (nm) of 90% @ harvest</b> | <b>Mean Particle Size (nm) @ 24 hrs after milling</b> | <b>Particle Size of 90% (nm) @ 24 hrs after milling</b> | <b>Mean Particle Size (nm) @ 1 week after milling</b> | <b>Particle size of 90% (nm) @ 1 week after milling</b> |
|-----------------|---------------------|------------------------|--|--|---|---|---|---|
| 1               | 4.0%                | 1% S1001               | 255<br>234                               | 378<br>353                                 |   |   | 297<br>247  | 436<br>372  |
| 2               | 2.5%                | 1% S1004               | 282<br>239                               | 381<br>341                                 | 284<br>275  | 382<br>370  | 232<br>217  | 329<br>207  |

These results demonstrate the ability to produce nanoparticulate insulin compositions which maintain stability and perhaps even improve upon storage using high energy milling techniques. Most importantly, the results demonstrate that, upon storage, little or no aggregation is observed. Thus, the data show the ability to consistently prepare nanoparticulate insulin formulations which maintain their integrity upon storage.

### Example 3

The purpose of this example is to examine nanoparticle insulin prepared in accordance with the invention and test the stability of the compositions over an extended storage period.

Nanoparticulate insulin was prepared in accordance with the procedure of Example 2. Milling was carried out until the insulin particles were found to have a mean particle size of 100 nm, with 90% of the particles less than 145 nm..

FIG. 1 shows SEM images of insulin particles before and after milling, clearly demonstrating the effect of the milling and, more importantly, the capacity of the surface stabilizers to prevent agglomeration of the insulin particles. Analysis of a sample of this material stored for six months at 5°C revealed 90% of the insulin particles to be smaller than 145 nm.

**Example 4**

The purpose of this example was to determine the insulin content of a nanoparticulate composition following milling, and to determine if the milling process results in loss of insulin.

Nanoparticulate insulin was prepared in accordance with the procedure of Example 1 from a slurry of 2% insulin having 1% Pluronic® F68 and 0.1% sodium deoxycholic acid as surface stabilizers.

Aliquots of the nanoparticulate insulin were centrifuged at 13,000 g for 15 min. The supernatant was isolated and the pellet washed in Phosphate Buffer Saline (PBS) and re-spun. The pellets and supernatant fractions post-centrifugation were subjected to analysis on an SDS-PAGE gel. Raw insulin in PBS additionally containing 0.1 M HCl for stability (Sigma I-5500) served as a control.

Reducing gels used in the analysis were prepared as 16% acrylamide gels and comprised 5.33 ml of acrylamide solution, 2.02 ml distilled water, 2.50 ml of 1.5 M TRIS buffer at pH of 8.8, 100  $\mu$ m 10% SDS, 50  $\mu$ m 10% ammonium persulfate, and 5  $\mu$ m of TEMED (N,N,N',N'-tetramethylethylene diamine). Reducing gels are useful for visualizing insulin bands, which appear as a dimer, tetramer, etc., and for identifying degraded insulin fragments.

The composition of the running buffer and loading buffers were as follows:

|   |        |  |        |
|---|--------|--|--------|
| <b>5X Electrode Buffer</b><br>(1X = 25 mM Tris, 192 mM Glycine, 0.1% SDS, pH 8.3) |        | <b>Sample Loading Buffer</b><br>(SDS reducing buffer: 62.5 mM Tris HCl (pH6.8), 20% Glycerol, 2% SDS, 5% $\beta$ -mercaptoethanol) |        |
| Tris Base   | 37.5g  | Distilled water  | 3.0 ml |
| Glycine   | 180.0g | 0.5M Tris-HCl (pH6.8)  | 1.0 ml |
| SDS   | 12.5g  | Glycerol   | 1.6 ml |
| Distilled Water   | 2.5L   | 10% SDS  | 1.6 ml |
| Dilute 200 ml of the 5X stock with 800 ml distilled water before use.             |        | $\beta$ -mercaptoethanol   | 0.4 ml |
|   |        | 0.5% (w/v) bromophenol blue (in water)   | 0.4 ml |

The samples and the supernatant were diluted to 1:10 and 1:50, respectively, with sample buffer and then heated to 95°C for 5 mins before loading onto the gel. The volume of

supernatant loaded was the amount that would be needed to load the required amount of sample, if the insulin were left in suspension.

Two reducing gels were prepared, and the samples were loaded onto the gels so that the pellet and supernatant fractions from each sample could be observed side by side. The results are shown in FIG. 2 and FIG. 3.

The reducing gel shown in FIG. 2 was loaded with the following:

- Lane 1: Raw Insulin Sample
- Lane 2: Sample supernatant 1:50 dilution;
- Lane 3: Sample supernatant 1:10 dilution;
- Lane 4: Pellet Sample 1:50 dilution;
- Lane 5: Pellet Sample 1:10 dilution; and
- Lane 6: High Molecular Weight markers.

The reducing gel shown in FIG. 3 was loaded with the following:

- Lane 1: High Molecular Weight markers;
- Lane 2: Pellet Sample 1:10 dilution;
- Lane 3: Pellet Sample 1:50 dilution;
- Lane 4: Raw Insulin Sample; and
- Lane 5: Raw Insulin Sample.

The gels shown in FIGs 2 and 3 demonstrate that the pellet fraction of the milled insulin formulation contains all of the insulin. The lanes loaded with supernatant appear to be free of insulin. This indicates that all of the insulin in the nanoparticulate insulin composition is entrapped within or associated with the nanoparticulate composition, with little or no insulin free in solution. Thus, there is essentially 100% entrapment or association of the insulin within the nanoparticulate composition.

#### Example 5

The purpose of this example was to test the effectiveness of nanoparticulate insulin compositions parenterally administered, and to compare the effectiveness of the formulations with conventional raw insulin compositions.

All animals (Wistar rats) were weighed and fasted for 4 hours prior to study initiation. The rats (250-300 g) were anaesthetized by intra-muscular (IM) administration of a solution of

ketamine (22.5 mg/animal) and acepromazine (0.75 mg/kg) one hour prior to administration of the test solutions. The sample of nanoparticulate insulin prepared in accordance with the procedure of Example 1 from a slurry of 2% insulin having 1% Pluronic® F68 and 0.1% sodium deoxycholic acid as surface stabilizers was formulated as a solution in PBS (phosphate buffer solution) pH 7.4. Groups of 6 animals received an amount of the solution sufficient to provide one IU of insulin by subcutaneous, intramuscular, or intraperitoneal injection in a randomized non-crossover study. Blood glucose values for the rats were measured using an Accutren® alpha glucometer (Boehringer Mannheim) following systemic blood samples that were taken from the tail artery. A baseline blood sample was taken and samples were taken at intervals post injection up to four hours. Anesthesia was maintained throughout the study. A non-formulated insulin solution was utilized as a reference.

The rat model used is a hyperglycemic model. The pharmacokinetic parameters of the test are given in FIG. 4. It can be seen from the results in FIG. 4 that the insulin compositions of the present invention are biologically active and at least approximately equal to the reference insulin solution. Fig. 5 illustrates the AUC ( $\mu\text{U}/\text{ml}\cdot\text{h}$ ) for the sample by the three modes of parenteral administration in comparison to the reference insulin solution. The analysis shown in FIG. 5 likewise establishes the equivalence of the test preparation to the reference insulin solution.

FIGs 6 and 7 are companion concentration-time profiles in that FIG 6 illustrates mean glucose concentration while FIG. 7 illustrates mean insulin concentration. As described above, both plot the test sample administered via subcutaneous, intramuscular and intraperitoneal injection in comparison to a reference insulin preparation. If an insulin preparation is biologically active, a fall in blood glucose levels is expected to coincide with the rise in blood insulin levels. The profiles in FIGs 6 and 7 clearly establish that the nanoparticulate insulin formulations of the invention are biologically active.

Overall, the results reported in the figures demonstrate that the nanoparticulate insulin compositions of the present invention have excellent bioactivity following parenteral administration, which is at least equivalent to the reference insulin solution tested. In the plot of change in blood glucose level plotted in FIG. 8, it can be seen that the nanoparticle preparation of the present invention is advantageous over the reference insulin solution in maintaining a



lowering of the blood glucose level over the duration of the test. A particular advantage can be seen from the comparison via intraperitoneal administration.

It will be apparent to those skilled in the art that various modifications and variations can be made in the methods and compositions of the present invention without departing from the spirit or scope of the invention. Thus, it is intended that the present invention covers the modifications and variations of this invention provided they come within the scope of the appended claims and their equivalents. The preceding examples further illustrate the invention and are not to be construed as limiting of the specification and claims in any way.

We claim:

1. A nanoparticulate composition comprising insulin particles and at least one non-crosslinked surface stabilizer adsorbed on the surface thereof, wherein the insulin particles have an effective average particle size of less than about 5 microns.
2. The composition of claim 1, wherein the effective average particle size of the insulin particles is selected from the group consisting of less than about 4 microns, less than about 3 microns, less than about 2 microns, less than about 1500 nm, less than about 1 micron, less than about 800 nm, less than about 700 nm, less than about 600 nm, less than about 500 nm, less than about 400 nm, less than about 300 nm, less than about 200 nm, less than about 100 nm, less than about 75 nm, and less than about 50 nm.
3. The composition of claim 1 or 2, wherein the concentration of insulin is selected from the group consisting of from about 99.5% to about 0.001%, from about 95% to about 0.1%, and from about 90% to about 0.5%, by weight, based on the total combined dry weight of the insulin particles and at least one surface stabilizer, not including other excipients.
4. The composition of any of claims 1-3, wherein the concentration of the at least one surface stabilizer is selected from the group consisting of from about 0.001 to about 99.5%, from about 0.1 to about 95%, and from about 0.5 to about 90%, by weight, based on the total combined dry weight of the insulin particles and at least one surface stabilizer, not including other excipients.
5. The composition of any of claims 1-4, wherein the composition further comprises one or more pharmaceutically acceptable excipients, carriers, or a combination thereof.
6. The composition of any of claims 1-5, wherein the composition is formulated for administration selected from the group consisting of oral, pulmonary, nasal, parenteral, rectal, local, buccal, and topical administration.
7. A dosage form comprising the nanoparticulate insulin composition of any of claims 1-6, wherein the dosage form is a solid, semi-solid, or liquid dosage formulation.

8. A dosage form comprising the nanoparticulate insulin composition of any of claims 1-7, wherein the dosage form is selected from the group consisting of controlled release formulations, solid dose fast melt formulations, aerosol formulations, lyophilized formulations, tablets, solid lozenge, capsules, powders, and liquids for injection.
9. A pharmaceutical composition for parenteral administration comprising the nanoparticulate insulin composition of any of claims 1-8 and a suitable, pharmaceutically acceptable excipient.
10. The composition of any of claims 1-9, wherein the insulin is in a form selected from the group consisting of crystalline particles, semi-crystalline particles, semi-amorphous particles, amorphous particles, and a mixture thereof.
11. The composition of any of claims 1-10, further comprising at least two surface stabilizers.
12. The composition of any of claims 1-11, wherein the at least one surface stabilizer is selected from the group consisting of a nonionic surface stabilizer, an anionic surface stabilizer, a cationic surface stabilizer, and an ionic surface stabilizer.
13. The composition of claim 12, wherein the at least one surface stabilizer is selected from the group consisting of cetyl pyridinium chloride, gelatin, casein, phosphatides, dextran, glycerol, gum acacia, cholesterol, tragacanth, stearic acid, stearic acid esters and salts, calcium stearate, glycerol monostearate, cetostearyl alcohol, cetomacrogol emulsifying wax, sorbitan esters, polyoxyethylene alkyl ethers, polyoxyethylene castor oil derivatives, polyoxyethylene sorbitan fatty acid esters, polyethylene glycols, dodecyl trimethyl ammonium bromide, polyoxyethylene stearates, colloidal silicon dioxide, phosphates, sodium dodecylsulfate, carboxymethylcellulose calcium, hydroxypropyl celluloses, hydroxypropyl methylcellulose, carboxymethylcellulose sodium, methylcellulose, hydroxyethylcellulose, hydroxypropylmethylcellulose phthalate, noncrystalline cellulose, magnesium aluminum silicate, triethanolamine, polyvinyl alcohol, polyvinylpyrrolidone, 4-(1,1,3,3-tetramethylbutyl)-phenol polymer with ethylene oxide and formaldehyde, poloxamers, poloxamines, a charged phospholipid, dimyristoyl phosphatidyl glycerol, dioctylsulfosuccinate, dialkylesters of sodium sulfosuccinic

acid, sodium lauryl sulfate, alkyl aryl polyether sulfonates, mixtures of sucrose stearate and sucrose distearate, triblock copolymers of the structure:  $-(\text{-PEO-})--(\text{-PBO-})--(\text{-PEO-})-$ , p-isononylphenoxypoly-(glycidol), decanoyl-N-methylglucamide; n-decyl  $\beta$ -D-glucopyranoside, n-decyl  $\beta$ -D-maltopyranoside, n-dodecyl  $\beta$ -D-glucopyranoside, n-dodecyl  $\beta$ -D-maltoside, heptanoyl-N-methylglucamide, n-heptyl- $\beta$ -D-glucopyranoside, n-heptyl  $\beta$ -D-thioglucoside, n-hexyl  $\beta$ -D-glucopyranoside, nonanoyl-N-methylglucamide, n-nonyl  $\beta$ -D-glucopyranoside, octanoyl-N-methylglucamide, n-octyl- $\beta$ -D-glucopyranoside, octyl  $\beta$ -D-thioglucopyranoside, lysozyme, a PEG derivatized phospholipid, PEG derivatized cholesterol, a PEG derivatized cholesterol derivative, PEG derivatized vitamin A, PEG derivatized vitamin E, and random copolymers of vinyl acetate and vinyl pyrrolidone.

14. The composition of claim 12, wherein the at least one cationic surface stabilizer is selected from the group consisting of a polymer, a biopolymer, a polysaccharide, a cellulosic, an alginate, a nonpolymeric compound, and a phospholipid.

15. The composition of claim 12, wherein the at least one surface stabilizer is selected from the group consisting of cationic lipids, benzalkonium chloride, sulfonium compounds, phosphonium compounds, quarternary ammonium compounds, benzyl-di(2-chloroethyl)ethylammonium bromide, coconut trimethyl ammonium chloride, coconut trimethyl ammonium bromide, coconut methyl dihydroxyethyl ammonium chloride, coconut methyl dihydroxyethyl ammonium bromide, decyl triethyl ammonium chloride, decyl dimethyl hydroxyethyl ammonium chloride, decyl dimethyl hydroxyethyl ammonium chloride bromide,  $\text{C}_{12-15}$ dimethyl hydroxyethyl ammonium chloride,  $\text{C}_{12-15}$ dimethyl hydroxyethyl ammonium chloride bromide, coconut dimethyl hydroxyethyl ammonium chloride, coconut dimethyl hydroxyethyl ammonium bromide, myristyl trimethyl ammonium methyl sulphate, lauryl dimethyl benzyl ammonium chloride, lauryl dimethyl benzyl ammonium bromide, lauryl dimethyl (ethenoxy)<sub>4</sub> ammonium chloride, lauryl dimethyl (ethenoxy)<sub>4</sub> ammonium bromide, N-alkyl ( $\text{C}_{12-18}$ )dimethylbenzyl ammonium chloride, N-alkyl ( $\text{C}_{14-18}$ )dimethyl-benzyl ammonium chloride, N-tetradecylidmethylbenzyl ammonium chloride monohydrate, dimethyl didecyl ammonium chloride, N-alkyl and ( $\text{C}_{12-14}$ ) dimethyl 1-naphthylmethyl ammonium chloride, trimethylammonium halide, alkyl-trimethylammonium salts, dialkyl-dimethylammonium salts, lauryl trimethyl ammonium chloride, ethoxylated alkyamidoalkyldialkylammonium salt, an

ethoxylated trialkyl ammonium salt, dialkylbenzene dialkylammonium chloride, N-didecyltrimethyl ammonium chloride, N-tetradecyldimethylbenzyl ammonium, chloride monohydrate, N-alkyl(C<sub>12-14</sub>) dimethyl 1-naphthylmethyl ammonium chloride, dodecyltrimethylbenzyl ammonium chloride, dialkyl benzenealkyl ammonium chloride, lauryl trimethyl ammonium chloride, alkylbenzyl methyl ammonium chloride, alkyl benzyl dimethyl ammonium bromide, C<sub>12</sub> trimethyl ammonium bromides, C<sub>15</sub> trimethyl ammonium bromides, C<sub>17</sub> trimethyl ammonium bromides, dodecylbenzyl triethyl ammonium chloride, polydiallyldimethylammonium chloride (DADMAC), dimethyl ammonium chlorides, alkyldimethylammonium halogenides, tricetyl methyl ammonium chloride, decyltrimethylammonium bromide, dodecyltriethylammonium bromide, tetradecyltrimethylammonium bromide, methyl trioctylammonium chloride, POLYQUAT 10™, tetrabutylammonium bromide, benzyl trimethylammonium bromide, choline esters, benzalkonium chloride, stearalkonium chloride compounds, cetyl pyridinium bromide, cetyl pyridinium chloride, halide salts of quaternized polyoxyethylalkylamines, MIRAPOL™, ALKAQUAT™, alkyl pyridinium salts; amines, amine salts, amine oxides, imide azolinium salts, protonated quaternary acrylamides, methylated quaternary polymers, cationic guar, polymethylmethacrylate trimethylammonium bromide, polyvinylpyrrolidone-2-dimethylaminoethyl methacrylate dimethyl sulfate, hexadecyltrimethyl ammonium bromide, poly (2-methacryloxyethyltrimethylammonium bromide) (S1001), poly(N-vinylpyrrolidone/2-dimethylaminoethyl methacrylate) di methylsulphate quaternary (S1002), and poly(2-methylacryloxyamidopropyltrimethylammonium chloride) (S1004).

16. A method of making a nanoparticulate insulin composition comprising contacting insulin particles with at least one surface stabilizer for a time and under conditions sufficient to provide a nanoparticulate insulin composition having an effective average particle size of less than about 5 microns.

17. The method of claim 16, wherein said contacting comprises milling.
18. The method of claim 17, wherein said milling is high energy milling.
19. The method of claim 17, wherein said milling comprises wet milling.

20. The method of claim 16, wherein said contacting comprises homogenizing.

21. The method of any of claims 16-20, wherein the effective average particle size of the insulin particles is selected from the group consisting of less than about 4 microns, less than about 3 microns, less than about 2 microns, less than about 1500 nm, less than about 1 micron, less than about 800 nm, less than about 700 nm, less than about 600 nm, less than about 500 nm, less than about 400 nm, less than about 300 nm, less than about 200 nm, less than about 100 nm, less than about 75 nm, and less than about 50 nm.

22. The method of any of claims 16-21, wherein the concentration of insulin is selected from the group consisting of from about 99.5% to about 0.001%, from about 95% to about 0.1%, and from about 90% to about 0.5%, by weight, based on the total combined dry weight of the insulin particles and at least one surface stabilizer, not including other excipients.

23. The method of any of claims 16-22, wherein the concentration of the at least one surface stabilizer is selected from the group consisting of from about 0.001 to about 99.5%, from about 0.1 to about 95%, and from about 0.5 to about 90%, by weight, based on the total combined dry weight of the insulin particles and at least one surface stabilizer, not including other excipients.

24. The method of any of claims 16-23, wherein the insulin is in a form selected from the group consisting of crystalline particles, semi-crystalline particles, semi-amorphous particles, amorphous particles, and a mixture thereof.

25. The method of any of claims 16-24, wherein the composition further comprises at least two surface stabilizers.

26. The method of any of claims 16-25, wherein the at least one surface stabilizer is selected from the group consisting of a nonionic surface stabilizer, an anionic surface stabilizer, a cationic surface stabilizer, and an ionic surface stabilizer.

27. The method of any of claims 26, wherein the at least one surface stabilizer is selected from the group consisting of cetyl pyridinium chloride, gelatin, casein, phosphatides, dextran, glycerol, gum acacia, cholesterol, tragacanth, stearic acid, stearic acid esters and salts,

calcium stearate, glycerol monostearate, cetostearyl alcohol, cetomacrogol emulsifying wax, sorbitan esters, polyoxyethylene alkyl ethers, polyoxyethylene castor oil derivatives, polyoxyethylene sorbitan fatty acid esters, polyethylene glycols, dodecyl trimethyl ammonium bromide, polyoxyethylene stearates, colloidal silicon dioxide, phosphates, sodium dodecylsulfate, carboxymethylcellulose calcium, hydroxypropyl celluloses, hydroxypropyl methylcellulose, carboxymethylcellulose sodium, methylcellulose, hydroxyethylcellulose, hydroxypropylmethyl-cellulose phthalate, noncrystalline cellulose, magnesium aluminum silicate, triethanolamine, polyvinyl alcohol, polyvinylpyrrolidone, 4-(1,1,3,3-tetramethylbutyl)-phenol polymer with ethylene oxide and formaldehyde, poloxamers, poloxamines, a charged phospholipid, dimyristoyl phosphatidyl glycerol, dioctylsulfosuccinate, dialkylesters of sodium sulfosuccinic acid, sodium lauryl sulfate, alkyl aryl polyether sulfonates, mixtures of sucrose stearate and sucrose distearate, triblock copolymers of the structure:  $-(\text{-PEO})--(\text{-PBO})--(\text{-PEO})-$ , p-isononylphenoxypoly-(glycidol), decanoyl-N-methylglucamide; n-decyl  $\beta$ -D-glucopyranoside, n-decyl  $\beta$ -D-maltopyranoside, n-dodecyl  $\beta$ -D-glucopyranoside, n-dodecyl  $\beta$ -D-maltoside, heptanoyl-N-methylglucamide, n-heptyl- $\beta$ -D-glucopyranoside, n-heptyl  $\beta$ -D-thioglucoside, n-hexyl  $\beta$ -D-glucopyranoside, nonanoyl-N-methylglucamide, n-nonyl  $\beta$ -D-glucopyranoside, octanoyl-N-methylglucamide, n-octyl- $\beta$ -D-glucopyranoside, octyl  $\beta$ -D-thioglucopyranoside, lysozyme, a PEG derivatized phospholipid, PEG derivatized cholesterol, a PEG derivatized cholesterol derivative, PEG derivatized vitamin A, PEG derivatized vitamin E, and random copolymers of vinyl acetate and vinyl pyrrolidone.

28. The method of claim 26, wherein the at least one cationic surface stabilizer is selected from the group consisting of a polymer, a biopolymer, a polysaccharide, a cellulosic, an alginate, a nonpolymeric compound, and a phospholipid.

29. The method of claim 26, wherein the at least one surface stabilizer is selected from the group consisting of cationic lipids, benzalkonium chloride, sulfonium compounds, phosphonium compounds, quaternary ammonium compounds, benzyl-di(2-chloroethyl)ethylammonium bromide, coconut trimethyl ammonium chloride, coconut trimethyl ammonium bromide, coconut methyl dihydroxyethyl ammonium chloride, coconut methyl dihydroxyethyl ammonium bromide, decyl triethyl ammonium chloride, decyl dimethyl dihydroxyethyl ammonium chloride, decyl dimethyl hydroxyethyl ammonium chloride bromide,

$C_{12-15}$ dimethyl hydroxyethyl ammonium chloride,  $C_{12-15}$ dimethyl hydroxyethyl ammonium chloride bromide, coconut dimethyl hydroxyethyl ammonium chloride, coconut dimethyl hydroxyethyl ammonium bromide, myristyl trimethyl ammonium methyl sulphate, lauryl dimethyl benzyl ammonium chloride, lauryl dimethyl benzyl ammonium bromide, lauryl dimethyl (ethenoxy)<sub>4</sub> ammonium chloride, lauryl dimethyl (ethenoxy)<sub>4</sub> ammonium bromide, N-alkyl ( $C_{12-18}$ )dimethylbenzyl ammonium chloride, N-alkyl ( $C_{14-18}$ )dimethyl-benzyl ammonium chloride, N-tetradecyldimethylbenzyl ammonium chloride monohydrate, dimethyl didecyl ammonium chloride, N-alkyl and ( $C_{12-14}$ ) dimethyl 1-naphthylmethyl ammonium chloride, trimethylammonium halide, alkyl-trimethylammonium salts, dialkyl-dimethylammonium salts, lauryl trimethyl ammonium chloride, ethoxylated alkyamidoalkyldialkylammonium salt, an ethoxylated trialkyl ammonium salt, dialkylbenzene dialkylammonium chloride, N-didecyl dimethyl ammonium chloride, N-tetradecyldimethylbenzyl ammonium, chloride monohydrate, N-alkyl( $C_{12-14}$ ) dimethyl 1-naphthylmethyl ammonium chloride, dodecyl dimethylbenzyl ammonium chloride, dialkyl benzenealkyl ammonium chloride, lauryl trimethyl ammonium chloride, alkylbenzyl methyl ammonium chloride, alkyl benzyl dimethyl ammonium bromide,  $C_{12}$  trimethyl ammonium bromides,  $C_{15}$  trimethyl ammonium bromides,  $C_{17}$  trimethyl ammonium bromides, dodecylbenzyl triethyl ammonium chloride, poly-diallyldimethylammonium chloride (DADMAC), dimethyl ammonium chlorides, alkyldimethylammonium halogenides, tricetyl methyl ammonium chloride, decyltrimethylammonium bromide, dodecyltriethylammonium bromide, tetradecyltrimethylammonium bromide, methyl trioctylammonium chloride, POLYQUAT 10™, tetrabutylammonium bromide, benzyl trimethylammonium bromide, choline esters, benzalkonium chloride, stearylalkonium chloride compounds, cetyl pyridinium bromide, cetyl pyridinium chloride, halide salts of quaternized polyoxyethylalkylamines, MIRAPOL™, ALKAQUAT™, alkyl pyridinium salts; amines, amine salts, amine oxides, imide azolinium salts, protonated quaternary acrylamides, methylated quaternary polymers, cationic guar, polymethylmethacrylate trimethylammonium bromide, polyvinylpyrrolidone-2-dimethylaminoethyl methacrylate dimethyl sulfate, hexadecyltrimethyl ammonium bromide, poly (2-methacryloxyethyltrimethylammonium bromide) (S1001), poly(N-vinylpyrrolidone/2-dimethylaminoethyl methacrylate) di methylsulphate quaternary (S1002), and poly(2-methylacryloxyamidopropyltrimethylammonium chloride) (S1004).



30. Use of a nanoparticulate insulin composition for the manufacture of a medicament suitable for administration to a n animal, including a mammal, in need, wherein the composition comprises:

- (a) insulin particles having an effective average particle size of less than about 5 microns prior to inclusion in the dosage form; and
- (b) at least one surface stabilizer adsorbed on the surface of the insulin particles.

31. The use of claim 30, wherein the effective average particle size of the insulin particles is selected from the group consisting of less than about 4 microns, less than about 3 microns, less than about 2 microns, less than about 1500 nm, less than about 1 micron, less than about 800 nm, less than about 700 nm, less than about 600 nm, less than about 500 nm, less than about 400 nm, less than about 300 nm, less than about 200 nm, less than about 100 nm, less than about 75 nm, and less than about 50 nm.

32. The use of claim 30 or 31, wherein the concentration of insulin is selected from the group consisting of from about 99.5% to about 0.001%, from about 95% to about 0.1%, and from about 90% to about 0.5%, by weight, based on the total combined dry weight of the insulin particles and at least one surface stabilizer, not including other excipients.

33. The use of any of claims 30-32, wherein the concentration of the at least one surface stabilizer is selected from the group consisting of from about 0.001 to about 99.5%, from about 0.1 to about 95%, and from about 0.5 to about 90%, by weight, based on the total combined dry weight of the insulin particles and at least one surface stabilizer, not including other excipients.

34. The use of any of claims 30-33, wherein the insulin is in a form selected from the group consisting of crystalline particles, semi-crystalline particles, semi-amorphous particles, amorphous particles, and a mixture thereof.

35. The use of any of claims 30-34, wherein the composition further comprises at least two surface stabilizers.

36. The use of any of claims 30-35, wherein the at least one surface stabilizer is selected from the group consisting of a nonionic surface stabilizer, an anionic surface stabilizer, a cationic surface stabilizer, and an ionic surface stabilizer.

37. The use of claim 36, wherein the at least one surface stabilizer is selected from the group consisting of cetyl pyridinium chloride, gelatin, casein, phosphatides, dextran, glycerol, gum acacia, cholesterol, tragacanth, stearic acid, stearic acid esters and salts, calcium stearate, glycerol monostearate, cetostearyl alcohol, cetomacrogol emulsifying wax, sorbitan esters, polyoxyethylene alkyl ethers, polyoxyethylene castor oil derivatives, polyoxyethylene sorbitan fatty acid esters, polyethylene glycols, dodecyl trimethyl ammonium bromide, polyoxyethylene stearates, colloidal silicon dioxide, phosphates, sodium dodecylsulfate, carboxymethylcellulose calcium, hydroxypropyl celluloses, hydroxypropyl methylcellulose, carboxymethylcellulose sodium, methylcellulose, hydroxyethylcellulose, hydroxypropylmethyl-cellulose phthalate, noncrystalline cellulose, magnesium aluminum silicate, triethanolamine, polyvinyl alcohol, polyvinylpyrrolidone, 4-(1,1,3,3-tetramethylbutyl)-phenol polymer with ethylene oxide and formaldehyde, poloxamers, poloxamines, a charged phospholipid, dimyristoyl phosphatidyl glycerol, dioctylsulfosuccinate, dialkylesters of sodium sulfosuccinic acid, sodium lauryl sulfate, alkyl aryl polyether sulfonates, mixtures of sucrose stearate and sucrose distearate, triblock copolymers of the structure:  $-(\text{-PEO-})--(\text{-PBO-})--(\text{-PEO-})-$ , p-isononylphenoxypoly-(glycidol), decanoyl-N-methylglucamide; n-decyl  $\beta$ -D-glucopyranoside, n-decyl  $\beta$ -D-maltopyranoside, n-dodecyl  $\beta$ -D-glucopyranoside, n-dodecyl  $\beta$ -D-maltoside, heptanoyl-N-methylglucamide, n-heptyl- $\beta$ -D-glucopyranoside, n-heptyl  $\beta$ -D-thioglucoside, n-hexyl  $\beta$ -D-glucopyranoside, nonanoyl-N-methylglucamide, n-nonyl  $\beta$ -D-glucopyranoside, octanoyl-N-methylglucamide, n-octyl- $\beta$ -D-glucopyranoside, octyl  $\beta$ -D-thioglucopyranoside, lysozyme, a PEG derivatized phospholipid, PEG derivatized cholesterol, a PEG derivatized cholesterol derivative, PEG derivatized vitamin A, PEG derivatized vitamin E, and random copolymers of vinyl acetate and vinyl pyrrolidone.

38. The use of claim 36, wherein the at least one cationic surface stabilizer is selected from the group consisting of a polymer, a biopolymer, a polysaccharide, a cellulosic, an alginate, a nonpolymeric compound, and a phospholipid.

39. The use of claim 36, wherein the at least one surface stabilizer is selected from the group consisting of cationic lipids, benzalkonium chloride, sulfonium compounds, phosphonium compounds, quarternary ammonium compounds, benzyl-di(2-chloroethyl)ethylammonium bromide, coconut trimethyl ammonium chloride, coconut trimethyl ammonium bromide, coconut methyl dihydroxyethyl ammonium chloride, coconut methyl dihydroxyethyl ammonium bromide, decyl triethyl ammonium chloride, decyl dimethyl hydroxyethyl ammonium chloride, decyl dimethyl hydroxyethyl ammonium chloride bromide, C<sub>12-15</sub>dimethyl hydroxyethyl ammonium chloride, C<sub>12-15</sub>dimethyl hydroxyethyl ammonium chloride bromide, coconut dimethyl hydroxyethyl ammonium chloride, coconut dimethyl hydroxyethyl ammonium bromide, myristyl trimethyl ammonium methyl sulphate, lauryl dimethyl benzyl ammonium chloride, lauryl dimethyl benzyl ammonium bromide, lauryl dimethyl (ethenoxy)<sub>4</sub> ammonium chloride, lauryl dimethyl (ethenoxy)<sub>4</sub> ammonium bromide, N-alkyl (C<sub>12-18</sub>)dimethylbenzyl ammonium chloride, N-alkyl (C<sub>14-18</sub>)dimethyl-benzyl ammonium chloride, N-tetradecyldimethylbenzyl ammonium chloride monohydrate, dimethyl didecyl ammonium chloride, N-alkyl and (C<sub>12-14</sub>) dimethyl 1-naphthylmethyl ammonium chloride, trimethylammonium halide, alkyl-trimethylammonium salts, dialkyl-dimethylammonium salts, lauryl trimethyl ammonium chloride, ethoxylated alkyamidoalkyldialkylammonium salt, an ethoxylated trialkyl ammonium salt, dialkylbenzene dialkylammonium chloride, N-didecyldimethyl ammonium chloride, N-tetradecyldimethylbenzyl ammonium, chloride monohydrate, N-alkyl(C<sub>12-14</sub>) dimethyl 1-naphthylmethyl ammonium chloride, dodecyldimethylbenzyl ammonium chloride, dialkyl benzenealkyl ammonium chloride, lauryl trimethyl ammonium chloride, alkylbenzyl methyl ammonium chloride, alkyl benzyl dimethyl ammonium bromide, C<sub>12</sub> trimethyl ammonium bromides, C<sub>15</sub> trimethyl ammonium bromides, C<sub>17</sub> trimethyl ammonium bromides, dodecylbenzyl triethyl ammonium chloride, poly-diallyldimethylammonium chloride (DADMAC), dimethyl ammonium chlorides, alkyldimethylammonium halogenides, tricetyl methyl ammonium chloride, decyltrimethylammonium bromide, dodecyltriethylammonium bromide, tetradecyltrimethylammonium bromide, methyl trioctylammonium chloride, POLYQUAT 10™, tetrabutylammonium bromide, benzyl trimethylammonium bromide, choline esters, benzalkonium chloride, stearylalkonium chloride compounds, cetyl pyridinium bromide, cetyl pyridinium chloride, halide salts of quaternized polyoxyethylalkylamines, MIRAPOL™,

ALKAQUAT™, alkyl pyridinium salts; amines, amine salts, amine oxides, imide azolinium salts, protonated quaternary acrylamides, methylated quaternary polymers, cationic guar, polymethylmethacrylate trimethylammonium bromide, polyvinylpyrrolidone-2-dimethylaminoethyl methacrylate dimethyl sulfate, hexadecyltrimethyl ammonium bromide, poly (2-methacryloxyethyltrimethylammonium bromide) (S1001), poly(N-vinylpyrrolidone/2-dimethylaminoethyl methacrylate) di methylsulphate quaternary (S1002), and poly(2-methylacryloxyamidopropyltrimethylammonium chloride) (S1004).

FIGURE 1

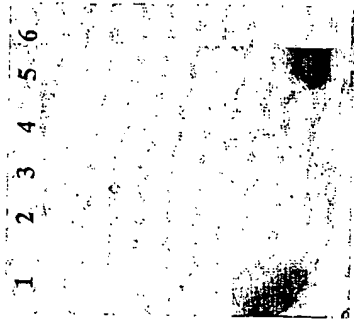
SEM Images (magnification x5000)



After Milling

Before Milling

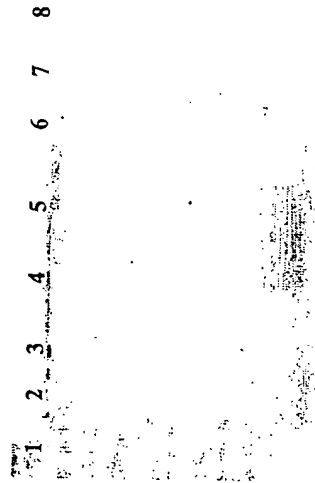
Figure 2



**Reducing Gel 1**

- Lane 1: Raw Insulin
- Lane2: Nano-insulin supernatant 1:50
- Lane3: Nano-insulin supernatant 1:10
- Lane4: Nano-insulin sample 1:50
- Lane5: Nano-insulin sample 1:10
- Lane6: High Molecular Weight markers

Figure 3

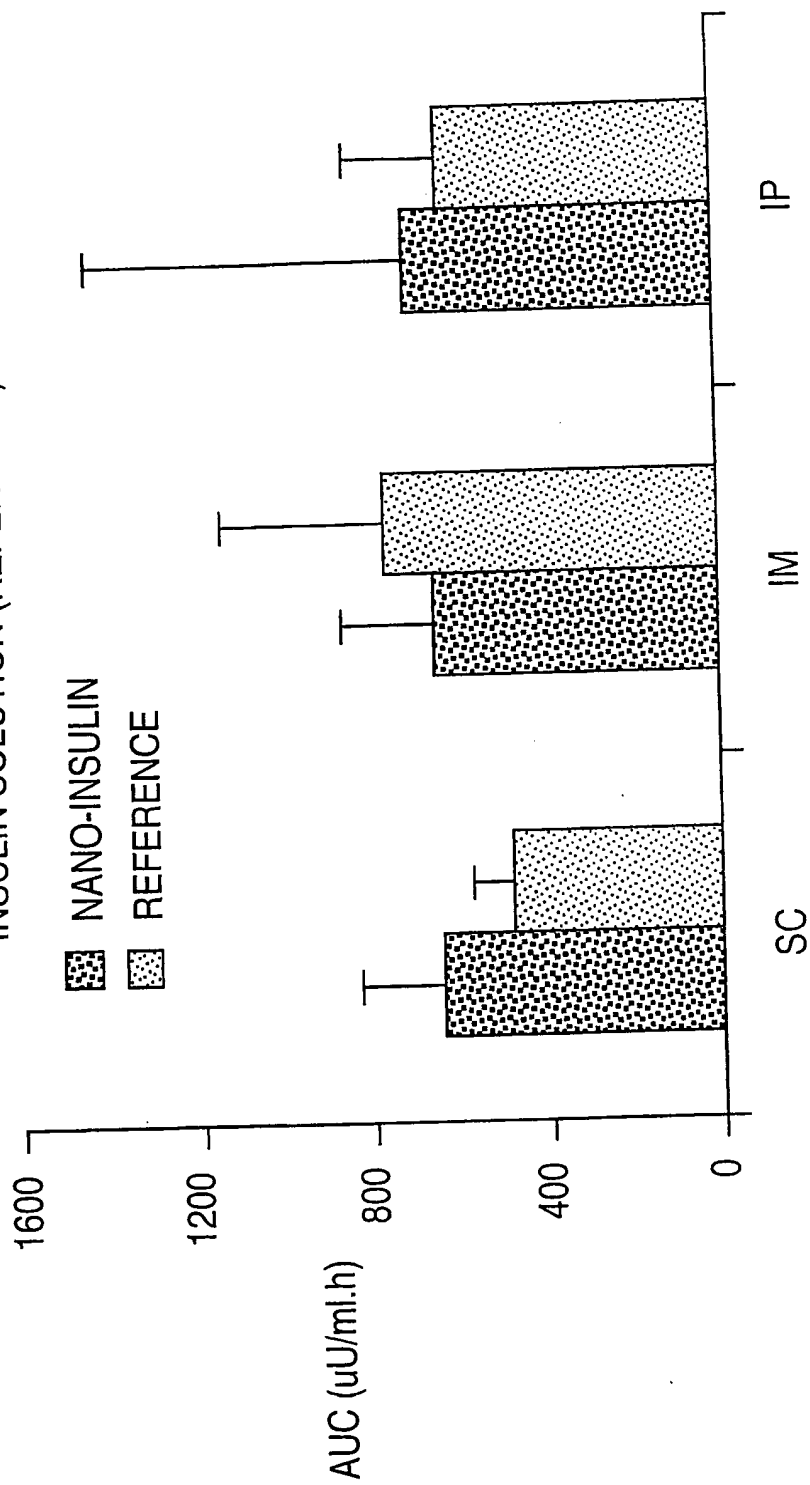


**Reducing Gel 2**

- Lane 1: High Molecular Weight markers
- Lane2: Nano-insulin pellet 1:10
- Lane3: Nano-insulin pellet 1:50
- Lane4: Raw insulin sample
- Lane5: Raw insulin sample
- Lane6: Empty
- Lane7: Empty
- Lane8: Empty

| FIG. 4                                     |              |                  |
|--|--------------|------------------|
| PHARMACOKINETIC PARAMETERS OF NANO-INSULIN |              |                  |
| ROUTE OF INJECTION                         | NANO-INSULIN | INSULIN SOLUTION |
| INTRAMUSCULAR                              |              |                  |
| • AUC (uU/ml.h)                            | 660 +/- 217  | 763 +/- 383      |
| • Cmax (uU/ml)                             | 569 +/- 136  | 664 +/- 175      |
| • Tmax (h)                                 | 0.4 +/- 0.1  | 0.8 +/- 0.6      |
| SUBCUTANEOUS                               |              |                  |
| • AUC (uU/ml.h)                            | 645 +/- 191  | 487 +/- 89       |
| • Cmax (uU/ml)                             | 453 +/- 129  | 417 +/- 79       |
| • Tmax (h)                                 | 0.5 +/- 0.2  | 0.3 +/- 0.1      |
| INTRAPERITONEAL                            |              |                  |
| • AUC (uU/ml.h)                            | 720 +/- 745  | 639 +/- 216      |
| • Cmax (uU/ml)                             | 655 +/- 799  | 390 +/- 113      |
| • Tmax (h)                                 | 1.5 +/- 1.4  | 0.6 +/- 0.1      |

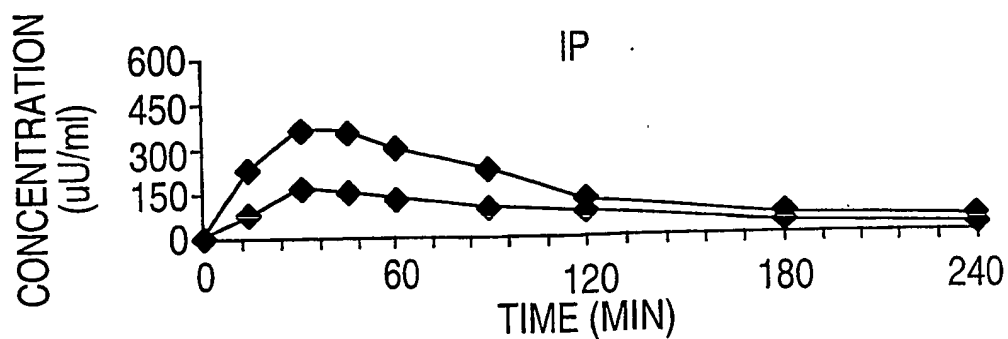
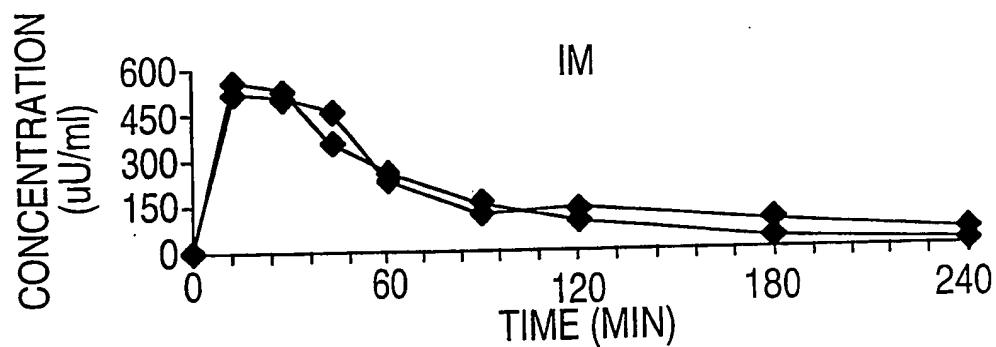
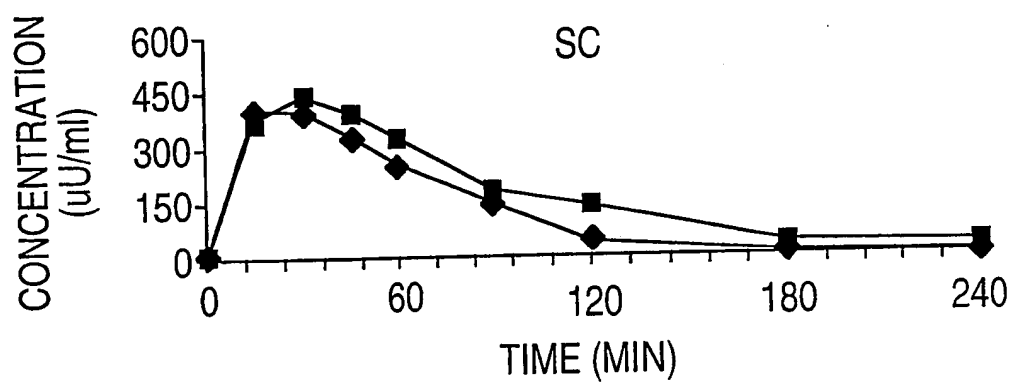
**FIG. 5**  
COMPARATIVE AUC'S OF NANO-INSULIN VS  
INSULIN SOLUTION (REFERENCE)





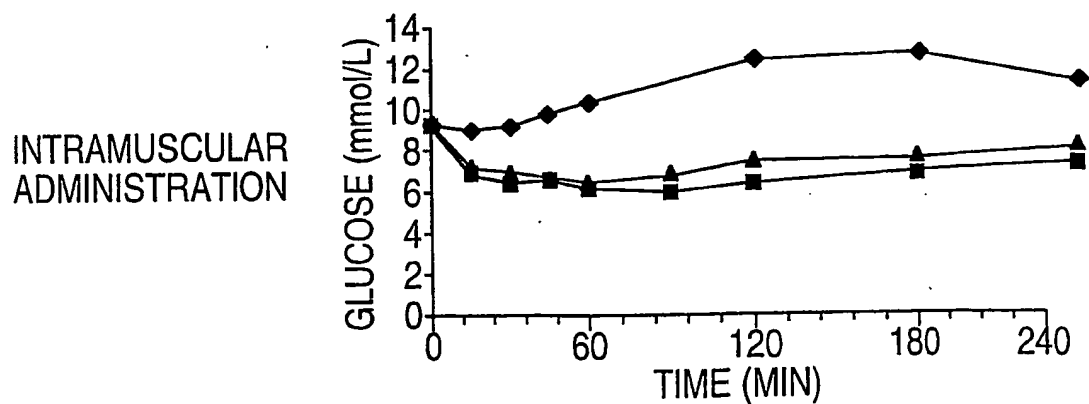
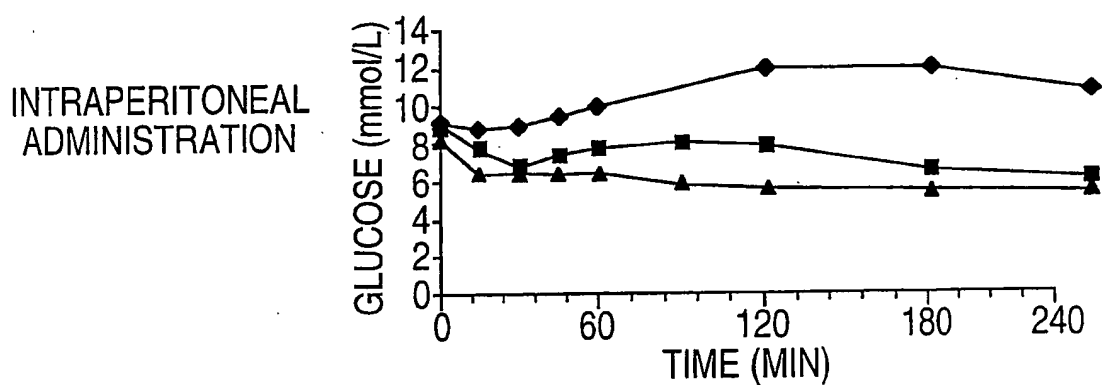
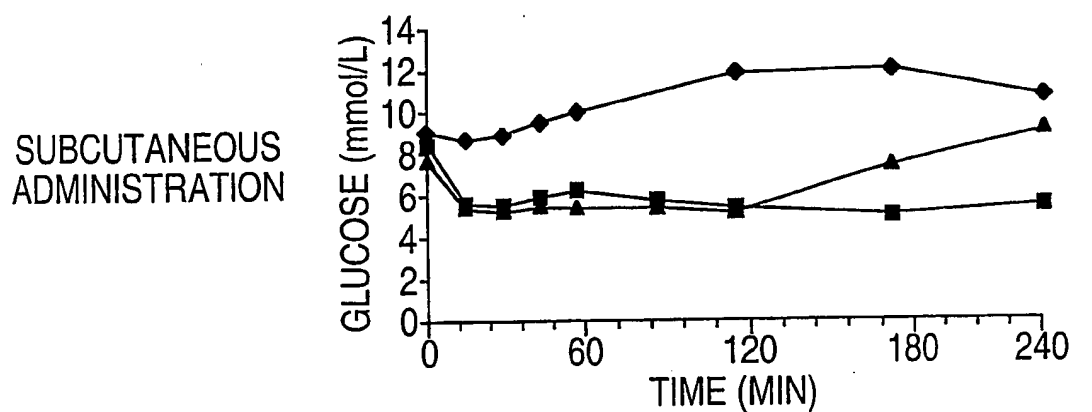
**FIG. 6**

INSULIN LEVELS IN BLOOD FOLLOWING ADMINISTRATION  
OF NANO-INSULIN (■) VS INSULIN SOLUTION (◆)



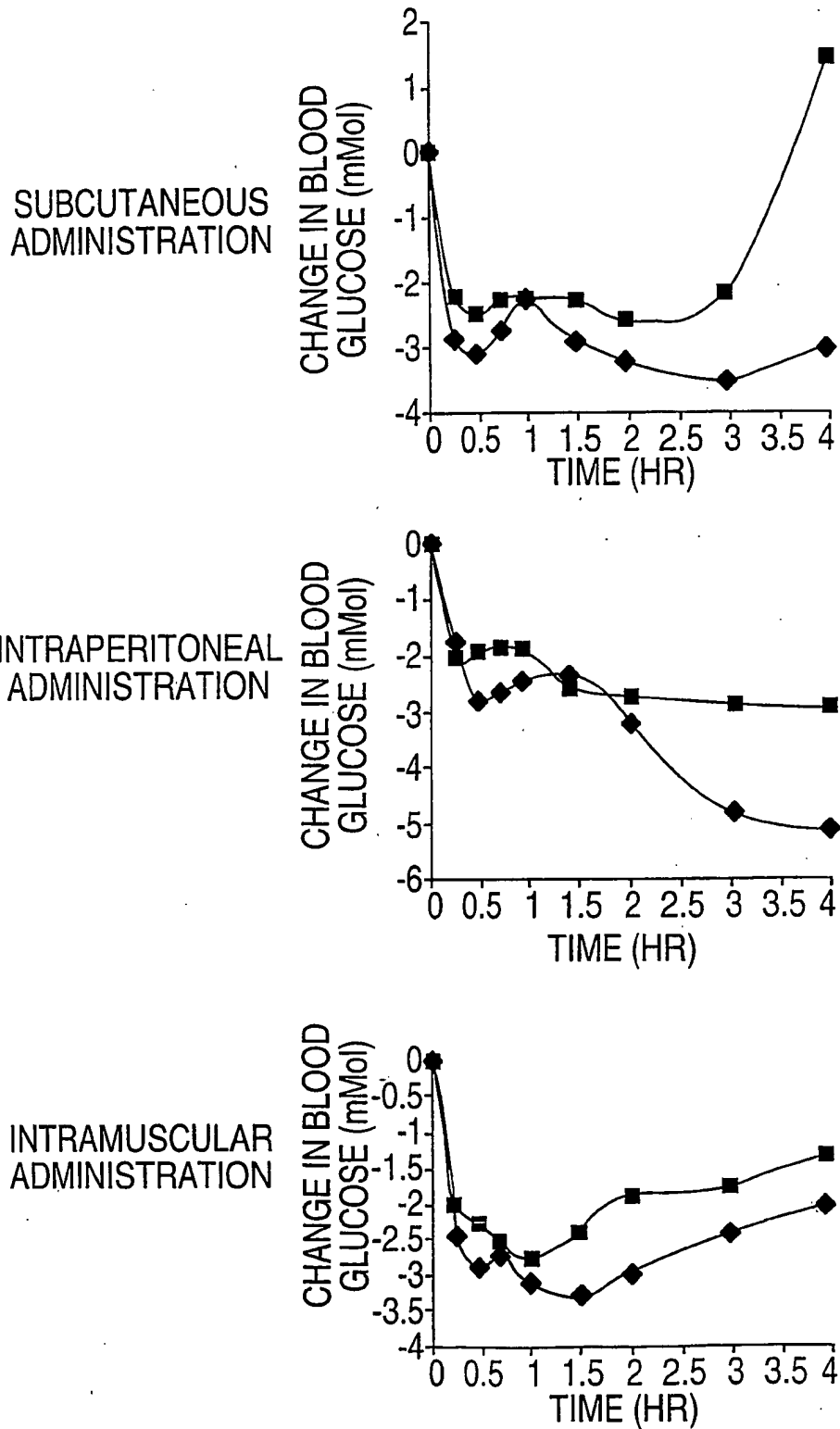
**FIG. 7**

BLOOD GLUCOSE LEVELS FOLLOWING THE ADMINISTRATION OF  
NANO-INSULIN (■); INSULIN SOLUTION (▲); CONTROL - NO INSULIN (◆)



**FIG. 8**

CHANGE IN BLOOD GLUCOSE LEVELS  
FOLLOWING ADMINISTRATION OF  
NANO-INSULIN (◆) VS INSULIN SOLUTION (■)



## INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 02/29679

A. CLASSIFICATION OF SUBJECT MATTER  
 IPC 7 A61K9/14 A61K38/28

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)  
 IPC 7 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, WPI Data

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category * | Citation of document, with indication, where appropriate, of the relevant passages  | Relevant to claim No. |
|------------|---|-----------------------|
| Y          | WO 00 27363 A (NANOSYSTEMS)<br>18 May 2000 (2000-05-18)<br>page 1, line 22<br>page 7, line 1 - line 4<br>page 13, line 1 - line 12<br>page 18, line 17 - line 25<br>page 24, line 29 - page 26, line 4<br>page 26, line 10 - page 28, line 9<br>---<br>-/-- | 1-39                  |

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

## \* Special categories of cited documents:

- \*A\* document defining the general state of the art which is not considered to be of particular relevance
- \*E\* earlier document but published on or after the international filing date
- \*L\* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- \*O\* document referring to an oral disclosure, use, exhibition or other means
- \*P\* document published prior to the international filing date but later than the priority date claimed

- \*T\* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- \*X\* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- \*Y\* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- \*G\* document member of the same patent family

Date of the actual completion of the international search

12 December 2002

Date of mailing of the international search report

19/12/2002

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2  
 NL - 2280 HV Rijswijk  
 Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,  
 Fax: (+31-70) 340-3016

Authorized officer

Giacobbe, S

## INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 02/29679

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

| Category * | Citation of document, with indication, where appropriate, of the relevant passages  | Relevant to claim No. |
|------------|---|-----------------------|
| Y          | <p>US 6 267 989 B1 (LIVERSIDGE ELAINE ET AL)<br/> 31 July 2001 (2001-07-31)<br/> column 6, line 45 - line 51<br/> column 6, line 60 -column 7, line 3<br/> column 7, line 13<br/> column 7, line 16<br/> column 7, line 38 -column 8, line 28<br/> column 8, line 37 - line 54<br/> column 8, line 55 -column 9, line 14<br/> column 9, line 18 - line 67<br/> column 10, line 1 - line 67<br/> claims 1-14</p> | 1-39                  |
| Y          | <p>WO 99 02665 A (NANOSYSTEMS LLC)<br/> 21 January 1999 (1999-01-21)<br/> page 2, last paragraph<br/> page 5, paragraph 4<br/> page 10, paragraph 2 -page 11, paragraph 3<br/> page 12, paragraph 2 -page 13, paragraph 2<br/> page 16, paragraph 3</p>   | 1-39                  |
| Y          | <p>US 5 470 583 A (RAJAGOPALAN NATARAJAN ET AL)<br/> 28 November 1995 (1995-11-28)<br/> the whole document</p>  | 1-39                  |
| Y          | <p>EP 0 499 299 A (STERLING WINTHROP INC)<br/> 19 August 1992 (1992-08-19)<br/> page 3, line 41 - line 42<br/> page 4, line 13 -page 5, line 25<br/> page 5, line 27<br/> page 6, line 5 - line 18<br/> page 6, line 42 - line 47</p>   | 1-39                  |
| Y          | <p>WO 00 51572 A (LIVERSIDGE ELAINE<br/> ;NANOSYSTEMS (US); GOTTARDY GRETA A (US))<br/> 8 September 2000 (2000-09-08)<br/> page 10, line 24 -page 11, line 20<br/> page 11, line 27 -page 14, line 10<br/> page 14, line 12 - line 23<br/> page 14, line 25 -page 15, line 10<br/> page 15, line 12 - line 25<br/> page 16, line 10 -page 17, line 27</p>   | 1-39                  |
| Y          | <p>WO 01 26635 A (ELAN PHARMA INTERNAT LTD<br/> ;BOSCH H WILLIAM (US); COOPER EUGENE R (U)<br/> 19 April 2001 (2001-04-19)<br/> the whole document</p>  | 1-39                  |

-/--

## INTERNATIONAL SEARCH REPORT

International Application No.

PCT/US 02/29679

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

| Category * | Citation of document, with indication, where appropriate, of the relevant passages  | Relevant to claim No. |
|------------|---|-----------------------|
| P,Y        | US 6 316 029 B1 (CUMMING KENNETH IAIN ET AL) 13 November 2001 (2001-11-13)<br>column 5, line 44 - line 49<br>column 5, line 58 - line 64<br>column 6, line 55,57<br>column 7, line 17 -column 8, line 19<br>column 8, line 20 - line 40<br>column 8, line 41 -column 9, line 55<br>column 9, line 56 - line 67<br>column 10, line 4 - line 17<br>column 10, line 53 -column 11, line 4<br>--- | 1-39                  |
| P,Y        | US 6 375 986 B1 (RUDDY STEPHEN B ET AL)<br>23 April 2002 (2002-04-23)<br>the whole document<br>---  | 1-39                  |
| E          | US 2002/110597 A1 (RUDDY STEPHEN B ET AL)<br>15 August 2002 (2002-08-15)<br>the whole document<br>-----   | 1-39                  |

## INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/US 02/29679

| Patent document<br>cited in search report |    | Publication<br>date | Patent family<br>member(s)   | Publication<br>date  |
|---|----|---------------------|--|--|
| WO 0027363                                | A  | 18-05-2000          | US 2002102294 A1<br>AU 1346900 A<br>EP 1128814 A1<br>JP 2002529396 T<br>WO 0027363 A1  | 01-08-2002<br>29-05-2000<br>05-09-2001<br>10-09-2002<br>18-05-2000   |
| US 6267989                                | B1 | 31-07-2001          | AU 3230600 A<br>EP 1161229 A1<br>JP 2002538199 A<br>WO 0053164 A1  | 28-09-2000<br>12-12-2001<br>12-11-2002<br>14-09-2000   |
| WO 9902665                                | A  | 21-01-1999          | US 6045829 A<br>AU 8183198 A<br>EP 1002065 A1<br>JP 2001509518 T<br>WO 9902665 A1<br>US 6221400 B1   | 04-04-2000<br>08-02-1999<br>24-05-2000<br>24-07-2001<br>21-01-1999<br>24-04-2001   |
| US 5470583                                | A  | 28-11-1995          | US 5336507 A<br>AU 662453 B2<br>AU 5046893 A<br>CA 2102267 A1<br>CZ 9302602 A3<br>EP 0601618 A2<br>FI 935305 A<br>HU 65758 A2<br>JP 6211646 A<br>NO 934204 A<br>NZ 250062 A<br>SK 139093 A3  | 09-08-1994<br>31-08-1995<br>23-06-1994<br>12-06-1994<br>15-06-1994<br>15-06-1994<br>12-06-1994<br>28-07-1994<br>02-08-1994<br>13-06-1994<br>27-04-1995<br>07-12-1994   |
| EP 0499299                                | A  | 19-08-1992          | US 5145684 A<br>AT 184202 T<br>AT 195416 T<br>AU 642066 B2<br>AU 1014592 A<br>AU 654836 B2<br>AU 1014792 A<br>CA 2059431 A1<br>CA 2059432 A1<br>DE 69229925 D1<br>DE 69229925 T2<br>DE 69231345 D1<br>DE 69231345 T2<br>DK 498482 T3<br>DK 499299 T3<br>EP 0498482 A2<br>EP 0499299 A2<br>ES 2139586 T3<br>ES 2149164 T3<br>FI 920321 A<br>FI 920322 A<br>GR 3032059 T3<br>GR 3034759 T3<br>HU 62462 A2<br>HU 60635 A2<br>IE 920217 A1<br>IE 920218 A1 | 08-09-1992<br>15-09-1999<br>15-09-2000<br>07-10-1993<br>30-07-1992<br>24-11-1994<br>30-07-1992<br>26-07-1992<br>26-07-1992<br>14-10-1999<br>17-02-2000<br>21-09-2000<br>26-04-2001<br>27-03-2000<br>02-01-2001<br>12-08-1992<br>19-08-1992<br>16-02-2000<br>01-11-2000<br>26-07-1992<br>26-07-1992<br>31-03-2000<br>28-02-2001<br>28-05-1993<br>28-10-1992<br>29-07-1992<br>29-07-1992 |

## INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/US 02/29679

| Patent document<br>cited in search report |    | Publication<br>date | Patent family<br>member(s) | Publication<br>date |
|---|----|---------------------|----------------------------|---------------------|
| EP 0499299                                | A  |                     | IL 100754 A                | 16-10-1996          |
|   |    |                     | IL 100755 A                | 08-12-1995          |
|   |    |                     | JP 4317053 A               | 09-11-1992          |
|   |    |                     | JP 4295420 A               | 20-10-1992          |
|   |    |                     | KR 200061 B1               | 15-06-1999          |
|   |    |                     | MX 9200291 A1              | 01-10-1992          |
|   |    |                     | MX 9200292 A1              | 01-10-1992          |
|   |    |                     | NO 920333 A                | 27-07-1992          |
|   |    |                     | NO 920334 A                | 27-07-1992          |
|   |    |                     | NZ 241361 A                | 25-06-1993          |
|   |    |                     | NZ 241362 A                | 25-06-1993          |
|   |    |                     | PT 499299 T                | 31-01-2001          |
|   |    |                     | SG 55104 A1                | 21-12-1998          |
|   |    |                     | RU 2074002 C1              | 27-02-1997          |
|   |    |                     | RU 2066553 C1              | 20-09-1996          |
|   |    |                     | US 5451393 A               | 19-09-1995          |
|   |    |                     | US 5494683 A               | 27-02-1996          |
|   |    |                     | US 5552160 A               | 03-09-1996          |
|   |    |                     | US 5399363 A               | 21-03-1995          |
|   |    |                     | US 5318767 A               | 07-06-1994          |
| WO 0051572                                | A  | 08-09-2000          | US 6270806 B1              | 07-08-2001          |
|   |    |                     | AU 3489600 A               | 21-09-2000          |
|   |    |                     | EP 1156788 A1              | 28-11-2001          |
|   |    |                     | JP 2002538099 A            | 12-11-2002          |
|   |    |                     | WO 0051572 A1              | 08-09-2000          |
| WO 0126635                                | A  | 19-04-2001          | US 2002068092 A1           | 06-06-2002          |
|   |    |                     | AU 7993800 A               | 23-04-2001          |
|   |    |                     | EP 1217993 A2              | 03-07-2002          |
|   |    |                     | WO 0126635 A2              | 19-04-2001          |
| US 6316029                                | B1 | 13-11-2001          | AU 6322801 A               | 26-11-2001          |
|   |    |                     | WO 0187264 A2              | 22-11-2001          |
| US 6375986                                | B1 | 23-04-2002          | AU 9501701 A               | 02-04-2002          |
|   |    |                     | WO 0224163 A1              | 28-03-2002          |
|   |    |                     | US 2002110597 A1           | 15-08-2002          |
| US 2002110597                             | A1 | 15-08-2002          | US 6375986 B1              | 23-04-2002          |
|   |    |                     | AU 9501701 A               | 02-04-2002          |
|   |    |                     | WO 0224163 A1              | 28-03-2002          |